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THE UNIVERSITY OF ALBERTA

THE INHIBITORY ACTIVITIES OF GOLD COORDINATION COMPLEXES IN THE TRANSDUCTION OF INFLAMMATORY STIMULI

BY

© JANICE ELENA RACHEL PARENTE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (MEDICINE)

EDMONTON, ALBERTA

SPRING, 1988

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THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Inhibitory Activites of Gold Coordination Complexes in the Transduction of Inflammatory Stimuli, submitted by Janice Elena Rachel Parente in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Supervisor

DATE: January 11, 1988

THIS THESIS IS DEDICATED

TO MY HUSBAND GEORGE

AND TO THE MEMBERS OF MY FAMILY

ABSTRACT

Gold coordination complexes are currently utilized as second-line, anti-rheumatic agents, although the mode by which they modulate the diseased state and promote remission in patients with chronic rheumatoid arthritis, remains uncertain. *In vitro*, gold compounds have been shown to display antiinflammatory properties toward the various cells involved in the inflammatory response. Of such cells, the neutrophil has been studied extensively. The gold compounds, auranofin and gold sodium thiomalate, varied markedly in their effects on the neutrophil. Auranofin suppressed the chemotactic peptide-induced activation of neutrophil degranulation and leukotriene production. In addition, auranofin inhibited the respiratory burst induced by chemotactic peptide-, fluoride anion-, or tumour-promoting phorbol ester-activation of neutrophils. Gold sodium thiomalate was much less potent an inhibitor.

Early signal transduction steps were not affected by auranofin since the mobilization of intracellular calcium stores was not inhibited. Tumour-promoting phorbol esters activate protein kinase C in a manner similar to endogenous diacylglycerol. The possiblity that auranofin may interfere with signal transduction at this level was investigated . Enzymatic assays

indicated that both auranofin and gold sodium thiomalate decreased the level of protein kinase C activity associated with the neutrophil cytosol. Phorbol ester-stimulated redistribution of protein kinase C activity from the cytosol to the membrane of the cell, a process associated with cellular activation, was inhibited by both gold compounds. Immunoblot analysis carried out using polyclonal anti-protein kinase C antibodies, revealed that the gold compounds did not promote degradation of protein kinase C and that more enzyme remained in the cytosol of cells co-treated with phorbol ester and gold compounds compared to phorbol ester alone. Further studies with intact cells indicated that endogenous protein phosphorylation mediated by protein kinase C was attenuated by auranofin but not by gold sodium thiomalate. However, both drugs inhibited neutrophil cytosolic and partially-purified platelet protein kinase C activities as well as the binding of phorbol ester to its receptor in these fractions. Current studies indicate that protein kinase C may be the target for the antiinflammatory activities of auranofin, and gold compounds in general.

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ABBREVIATIONS

A23187: calcium ionophore

ADP: adenosine diphosphate

ATP: adenosine triphosphate

ATPase: adenosine triphosphatase

Au: gold atom

$AuCl_4^-$: gold (III) chloride

AUR: auranofin

ß: beta

BSA: bovine serum albumin

C3b, C5a: cleavage products of the third and fifth components of complement, respectively

C567: complex of the fifth, sixth and seventh components of complement

C3bBb: complex of the third and B components of the alternative complement pathway

Ca^{2+}: calcium cation

$CaCl_2$: calcium chloride

$-CH_3$: methyl group

Cl^-: chloride anion

cm: centimetre

$-COO^-$: carboxyl group

cpm: counts per minute

C-SH: cytosol-associated thiol group

Cys: cysteine

°: degree

DEAE: diethylaminoethyl

DNA: deoxyribonucleic acid

EC: Enzyme Commission

EDTA: ethylenediaminetetraacetic acid

EFG: epidermal growth factor

EGTA: ethylene glycol bis (ß-aminoethyl ether)-N,N,-N',N'-tetraacetic acid

etc.: etcetera

F^-: fluoride anion

Fc: constant region of immunoglobulin

fMet-Leu-Phe: n-formylmethionyl-leucyl-phenylalanine

γ: gamma

GABA: gamma-aminobutyric acid

G-protein: guanine nucleotide-binding regulatory protein

GRF: granule rich fraction

GSG: gold sodium thioglucose

GST: gold sodium thiomalate

h: hour

H^+: hydrogen ion

^3H: tritium

H_2O_2: hydrogen peroxide

H-7: 1-(5-isoquinolinesulfonyl)-2-methylpiperazine

HBSS: Hanks' Balanced Salt Solution

HCl: hydrochloric acid

Hepes: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HLA: human-leukocyte-associated antigens

HMG CoA: high-mobility group coenzyme A

^{125}I: radioisotope of iodine

IC_{50}: 50 per cent inhibitory concentration

IgG: immunoglobulin G

IgE: immunoglobulin E

IL2: interleukin 2

K^+: potassium ion

KCl: potassium chloride

K_D: dissociation constant

kD: kilodalton

KH_2PO_4: potassium phosphate

K_i: dissociation constant in presence of inhibitor

L: litre

LDH: lactate dehydrogenase

log: logarithm

LTB_4: leukotriene B_4

LTC_4: leukotriene C_4

M: molar

mCi: millicurie

Me_2SO: dimethylsulfoxide

mg: milligram

µg: microgram

$MgCl_2$: magnesium chloride

$MgSO_4$: magnesium sulfate

min: minute

mL: millilitre

µL: microlitre

µm: micrometre

mM: millimolar

µM: micromolar

mmol: millimole

µmol: micromole

M_r: relative molecular mass

M-SH: membrane-associated thiol group

MWt: molecular weight

n: number of experiments

N: normal

-N=: nitrogen

Na^{2+}: sodium ion

NaCl: sodium chloride

NAD: nicotinamide adenine dinucleotide

$NADP^+$: nicotinamide adenine dinucleotide phosphate (oxidized)

NADPH: nicotinamide adenine dinucleotide phosphate (reduced)

NaF: sodium fluoride

$NaHCO_3$: sodium carbonate

Na_2HPO_4: sodium phosphate

ND: not determined

ng: nanogram

nm: nanometre

nM: nanomolar

nmol: nanomole

NP-40: Nonidet P-40

O_2: molecular oxygen

O_2^-: superoxide anion

OAG: 1-oleoyl-2-acetylglycerol

OH· : hydroxyl radical

OX^-: oxyhalide anion

π : pi

%: per cent

^{32}P: radioisotope of phosphorus

P47: 47,000 molecular weight protein

PAGE: polyacrylamide gel electrophoresis

PEt_3: triethylphosphine group

pg: picogram

PGE_2: prostaglandin E_2

pH: $-\log[H^+]$

pmol: picomole

PMSF: phenylmethylsulfonyl fluoride

s: second

S: sulfur

SDS: sodium dodecyl sulfate

SE: standard error of the mean

x g: times gravity

TATG: tetraacetylthioglucose

TCA: trichloroacetic acid

TEPG: triethylphosphine gold

TEMED: N,N,N',N'-tetramethylethylenediamine

TPA: 12-O-tetradecanoyl-phorbol-13-acetate

Tris: tris(hydroxymethyl)aminomethane

v: volume

w: weight

X: unknown amino acid

CHAPTER ONE

INTRODUCTION

I GOLD COORDINATION COMPLEXES

A. HISTORICAL BACKGROUND

In 1890, while searching for an effective chemotherapeutic agent against tubercle bacillus, Robert Koch discovered that gold cyanide inhibited the growth of this bacterium in vitro. Due to the cytotoxicity of gold compounds, less toxic compounds with therapeutic potential were eventually developed and applied to treat patients with active tuberculosis (Möllgaard, 1924). In comparing patients suffering from tuberculosis with others possessing characteristics of active rheumatoid arthritis, Forestier, in 1928, discovered that there were etiological, clinical and laboratory similarities between them. He conjectured that gold might prove to be effective in the treatment of rheumatoid arthritis since it was shown to be effective as a therapeutic measure against tuberculosis. With over five hundred treated rheumatoid arthritis patients, Forestier claimed 70 to 80% success with his patients on gold therapy (Forestier, 1934). Despite the fact that over fifty years of experience with gold coordination complexes as second-line therapy in the treatment of rheumatoid arthritis have clearly established their clinical efficacy, (Empire Rheumatism Council, 1961; Marshall,

1965; Cervini, 1977), the mode by which these compounds manifest changes in the diseased state remains uncertain and an area of ongoing investigation. In order to fully appreciate the mechanistics of gold drug action, an understanding of the chemistry of these compounds is necessary.

B. PHYSICAL AND CHEMICAL PROPERTIES OF GOLD COMPOUNDS

Until recently, very little progress had been made towards an understanding of the physical and chemical properties of gold compounds. Technological advances made in spectroscopy (nuclear magnetic resonance, infrared, Raman, and Mössbauer spectroscopy), have aided immensely in this field. According to studies of Sadler (1982), four basic properties of gold coordination complexes are important in understanding their mode of action: (1) the oxidation state of the gold atom; (2) the number and type of ligands coordinated to gold; (3) the geometry of ligands surrounding the gold atom; and (4) the thermodynamic and kinetic stability of the gold compound. Other properties, such as the partitioning between aqueous and non-aqueous phases (solubility), and charge of the complex, are likely to affect their uptake and distribution in biological systems.

The most common oxidation states of gold are I and

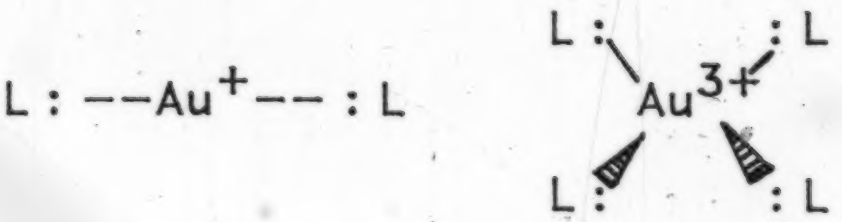
III, Au(I) and Au(III), although in aqueous solution Au(III) tends to be reduced to Au(I) in the absence of stabilizing ligands. When stabilized, Au(III) favours the formation of a square planar complex in which four ligands are arranged at 90° angles from one another relative to the gold nucleus. Au(I) generally forms linear, two coordination complexes in which the two ligands are positioned on opposite sides of the gold nucleus. Figure 1 demonstrates the coordination geometries of Au(I) and Au(III) complexes and lists examples of each.

Gold compounds in therapeutic usage are all Au(I) complexes (Sadler, 1976; Shaw, 1979; Brown and Smith, 1980). Au(III) complexes are strong oxidizing agents and are not used biologically due to their toxicity. For example, $AuCl_4^-$ will oxidize methionine residues in peptides to methionine sulfoxide, and cysteine to cystine and further to sulfonic acid. Au(I) can be stabilized by π-acceptor ligands, that is, ligands which donate electron density to Au(I) as well as accept electron density into their vacant π-orbitals. This type of arrangement provides extra stability to the Au(I) oxidation state. The ligands that bind to Au(I), in order of preference, are cyanide = cysteine-S = phosphines >> methionine-S-CH_3, histidine (=N-) > Cl^- >> $-COO^-$. Therefore, a thiolate such as

Figure 1: Coordination geometries of Au(I) and Au(III).

Au = gold atom

L: = ligand with lone-pair electrons



LINEAR

SQUARE PLANAR

eg: gold sodium thiomalate
gold sodium thioglucose
auranofin
gold chloride $AuCl_2^-$

gold chloride $AuCl_4^-$

cysteine will readily displace a Au(I)-bound methionine or a nitrogen from histidine.

Compounds which emerged in the 1920's with decreased toxicity compared to gold cyanide, yet therapeutically beneficial, were the water soluble gold sodium thiomalate (GST) and gold sodium thioglucose (GSG). Both of these compounds exist as polymers in solution where the Au(I) atom binds two sulfur atoms. Like GST and GSG, auranofin [AUR, (1-thio-β-D-glucopyranose 2,3,4,6-tetraacetate-S) (triethylphosphine) gold], a recently introduced oral gold compound, contains gold in the Au(I) state. The gold nucleus is coordinated to the tetraacetylthioglucose and triethylphosphine ligands. AUR does not polymerize in solution since the phosphine moiety does not participate in bridging (Sutton, 1972; Sadler, 1982; Sutton, 1983). The nature of its ligands also render AUR lipophilic.

C. THIOL-EXCHANGE REACTIONS OF GOLD COMPOUNDS

Various nuclear magnetic resonance spectroscopy titration experiments, using competitive thiol-containing compounds, have shown that Au(I) complexes can participate in thiol- or ligand-exchange reactions (Sadler, 1976; Shaw, 1979; Brown and Smith, 1981; Hempel and Mikuriya, 1981; Malik et al., 1981; Shaw, 1981; Sadler, 1982). This reaction is based on the ability of

the thiol-ligands attached to the Au(I) nucleus to be exchanged with competing thiols in its surroundings. Snyder and coworkers (1986) have studied the mechanism of gold, thiol-exchange in great detail using RAW 264.7 macrophages and labelled AUR either within the triethylphosphine (PEt_3), the gold, or the tetraacetylthioglucose (TATG) moieties of the molecule. They demonstrated that the gold moiety of AUR is taken up and distributed in these cells by a sequence of thiol-exchange reactions. In their model, cellular accumulation of the drug results from the sequential shuttling of Au(I)-PEt_3 or Au(I) moieties of the AUR molecule between cellular sulfhydryl groups. Cellular uptake, the rate limiting step, involves the displacement of TATG by membrane-associated thiol groups (M-SH):

$$TATG\text{-}Au\text{-}PEt_3 + M\text{-}SH \rightarrow TATG + M\text{-}S\text{-}Au\text{-}PEt_3$$

The rate of cellular association of any gold complex will depend on the reactivity of the leaving group. Thus, $Cl\text{-}Au\text{-}PEt_3$ associates more rapidly with cells than does AUR. Also, extracellular thiols such as albumin, cysteine etc., may compete with M-SH for the TATG ligating site of AUR and thereby reduce cellular association. Once associated with the cell, gold

complexes can be distributed intracellularly by the shuttling of M-S-Au-PEt_3 between cytosolic sulfhydryl groups (C-SH):

$$M\text{-}S\text{-}Au\text{-}PEt_3 + C\text{-}SH \rightarrow M\text{-}SH + C\text{-}S\text{-}Au\text{-}PEt_3$$

It should be noted that multiple intramembrane ligand exchanges of -Au-PEt_3 may occur prior to C-SH association. Bridging of cellular sulfhydryls may occur if the PEt_3 moiety is displaced by a suitable competitive ligand. Drug association with the cell by the sulfhydryl shuttle mechanism differs from traditional concepts of cellular uptake (Snyder et al., 1987a) and depends solely and simply on the chemical reactivities of the drug and cellular sulfhydryl-containing components.

The possible effects resulting from thiol-exchange by gold compounds are vast. Depending on the cell type, this process could lead to the disruption of membranes and membrane protein stucture and function. Once complexed to gold, it is possible that cytosolic proteins could be functionally altered.

D. NEUTROPHIL PHYSIOLOGY

Neutrophils belong to a series of terminally differentiated granulocytes of bone marrow origin.

Under normal conditions, neutrophils are found in the circulation where they comprise approximately 95% of the granulocytic component of human blood (Cronkite and Vincent, 1969). Tissue neutrophils arise in response to damage caused by irritation or infection via a well regulated process of migration known as chemotaxis. This process is initiated by the presentation to cells of a concentration gradient of a specific stimulus leading to the accumulation of cells at tissue sites of inflammation (Snyderman and Goetzyl, 1981). A host of substances generated during the inflammatory response has the capacity to both enhance the movement of neutrophils (chemokinesis), and to orient their movement in the direction of increasing concentration of the agent (chemotaxis). These stimuli also have the ability to alter the permeability of the endothelial lining of the post capillary venules to allow for extravasation of cells by a process termed diapedesis. A description of the various chemotactic factors is given in Table I.

Once localized to the site of insult, the main function of the neutrophil becomes one of recognition, phagocytosis and elimination of the pathogen. The recognition phase involves the binding of opsonized substances to specific receptors on the surface of the neutrophil. There are two major constituents of human serum, an immunoglobulin (IgG), and the third component

Table I: Factors chemotactic to human neutrophils.

Factor	Source	Reference
plasma kallikrein	kinin-generating sequence	Kaplan et al., 1972
C5a; C567	classic complement pathway	Fernandez et al., 1978; Lachmann et al., 1970
C3bBb	alternative complement pathway	Ruddy et al., 1975
fibrin fragments	fibrinolysis	Richardson et al., 1976
collagen fragments	collagenolysis	Postlethwaite and Kang, 1976
bacterial peptides and lipids	bacteria	Becker, 1972 Tainer et al., 1975
leukotriene B_4, 5- and 12-hydroxy eicosatetraenoic acid	arachidonate lipoxygenase	Goetzyl, 1983; Turk et al., 1982

of complement (C3b), known collectively as opsonins, which act upon certain foreign invaders to increase their palatability to phagocytic cells (Cline and Lehrer, 1968; Nelson, 1965). Neutrophils possess specific receptors for both the Fc portion of IgG (Messner and Jelinek, 1970; Boxer et al., 1978; Klempner and Gallin, 1978), and for fragments of the third component of complement, primarily C3b (Ehlenberger and Nussenzweig, 1977; Boxer et al., 1978). When contact is established between the neutrophil and the opsonized particle, the particle is then ingested by the cell by a process termed phagocytosis. In this process, the neutrophil extends pseudopodia from the site of particle attachment which eventually surrounds the particle and ultimately fuses at its distal pole (Moore et al., 1978). Following phagocytosis, membrane fusion leads to the discharge of lysosomal constituents (hydrolytic enzymes and antibacterial factors), into this phagocytic vacuole. Human neutrophils contain two major types of lysosomal granules: the azurophilic and the specific. Whereas the azurophilic granules contain myeloperoxidase, acid hydrolases such as ß-glucuronidase, and a limited supply of lysozyme, the specific granules are rich in lysozyme as well as containing a vitamin B_{12}-binding protein and lactoferrin (Gallin, 1984). A third class of small, gelatinase-containing

granules has been described (Dewald et al., 1982). Human neutrophils, therefore, contain granule-associated enzymes with the capacity to hydrolyze a wide variety of both natural and synthetic substrates. While the granular contents are normally discharged intracellularly into phagocytic vacuoles, under certain circumstances, they are released extracellularly, independently of phagocytosis. Thus, phagocytosis is not an absolute prerequisite for degranulation by neutrophils (Henson, 1972; Henson and Oades, 1975), and degranulation can be activated by soluble as well as particulate stimuli (Goldstein et al., 1974; Estensen et al., 1974; Goldstein et al., 1975a,b; Hoffstein et al., 1976; Goldstein et al., 1977).

Together with degranulation, the respiratory burst of neutrophils constitutes cellular events involved in the elimination of pathogenic material. The enzymatic system responsible for the respiratory burst is the NADPH oxidase first described by Rossi and Zatti in 1964 and Zatti and Rossi in 1966. The enzyme is located in the plasma membrane (Briggs et al., 1975; Dewald et al., 1979), and is activated during phagocytosis, as well as independently of phagocytosis, by various soluble stimuli. NADPH oxidase, completely dormant in unstimulated cells, catalyzes the oxidation of NADPH formed by glucose metabolism through the hexose

monophosphate shunt, according to the following reaction:

$$NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2O_2^- + 2H^+$$

Over the last few years, much data on the chemical composition of NADPH oxidase has been collected (Rossi, 1986), and it is generally agreed that a flavoprotein, a cytochrome (cytochrome b-558), and various quinones contribute to its structure. The arrangement of these components comprises an electron transport chain where the flavoprotein acts as NADPH dehydrogenase-cytochrome b reductase, with the possible participation of quinones. Cytochrome b-558 has been proposed to reduce O_2 to O_2^-.

The consumption of O_2 and production of O_2^- that occur during the respiratory burst are accounted for by NADPH oxidase activation. H_2O_2, as well as other active oxygen metabolites, are subsequently produced by reactions described in Figure 2. In concert, these active oxygen species (O_2^-, H_2O_2, $OH\cdot$, OX^-, etc.), have toxic effects on microorganisms and neoplastic tissue (Johnston et al., 1975). As foreign material is phagocytosed, the NADPH oxidase complex, located in the plasma membrane, becomes part of the phagocytic vacuole thereby releasing active oxygen

Figure 2: The major pathways for oxygen metabolism during the neutrophil respiratory burst.

a - NADPH oxidase catalyzes the one electron reduction of oxygen (O_2) to superoxide anion (O_2^-).

b - O_2^- is further reduced to hydrogen peroxide (H_2O_2).

c - H_2O_2 and O_2^- react to generate the hydroxyl radical ($OH\cdot$).

d - Myeloperoxidase catalyzes microbicidal reactions using H_2O_2 and halide ions (X^-) as substrate leading to halogenization of cellular and microbial proteins.

e - O_2^- escaping the phagocytic vacuole is reduced to H_2O_2 by superoxide dismutase.

f - Catalase reduces H_2O_2 to O_2 and water (H_2O).

g - NADPH is formed by the oxidation of glucose in the hexose monophosphate shunt.

cytosplasm
phagocytic vacuole
O_2
NADPH
a
OXIDASE
O_2^-
O_2^-
H^+
b
c
d
e
f
H_2O_2
H_2O_2
X^-
OX^-
$O_2 + OH^- + OH\cdot$
O_2
H_2O
NADPH
$NADP^+$
g

species within this space enabling destruction of the engulfed material (Cohen et al., 1980). Since phagocytosis is not a prerequisite for both degranulation and activation of the respiratory burst (Goetzyl and Austen, 1974; Hensen and Oades, 1975; Goldstein et al., 1975c; DeChatelet et al., 1976; Goldstein et al., 1976; Goldstein et al., 1977; Cohen and Chovaniec, 1978), cellular stimulation in the absence of a foreign agent can lead to the destruction of otherwise normal tissues.

E. IN VITRO ACTIVITIES OF GOLD COORDINATION COMPLEXES IN THE NEUTROPHIL

Gold coordination complexes have been shown to affect a variety of cells and cellular functions (review, Chaffman et al., 1984). However, the following will focus on the interaction of these compounds with normal neutrophil functioning.

Hafström and coworkers (1983a) studied neutrophil chemotaxis by preincubating cells with AUR followed by cellular stimulation with serum or bacterial factor cytotaxins. They observed an inhibitory effect with increasing dose of drug which could not be reversed by the removal of the drug by washing the cells. AUR did not inhibit random migration (chemokinesis) and GST was virtually ineffective. This same group of investigators

(Hafström et al., 1984a) utilized a soluble chemotactic stimulus, leukotriene B_4, and again observed AUR inhibition of stimulus-induced neutrophil chemotaxis. When activated for phagocytosis by zymosan particles (DiMartino and Walz, 1977), Candida albicans (Davis et al., 1982), yeast cells (Davis et al., 1982; Hafström et al., 1983b) or opsonized erythrocytes (Davis et al., 1982), neutrophils pretreated with AUR were significantly suppressed in this capacity compared to control cells. This effect was dependent on AUR dose and GST was shown to have minimal inhibitory activity.

The effect of gold compounds on both neutrophil degranulation and respiratory burst activities has been examined in detail by various groups. The consensus is that, whereas GST displayed little or no inhibitory activity on these cellular functions, AUR proved to be an effective inhibitor. Early studies of AUR's effect on lysosomal enzyme release in vitro utilized phagocytic neutrophil stimuli. During phagocytosis of zymosan particles, DiMartino and Walz (1977) showed that AUR produced a dose-dependent reduction of both β-glucuronidase and lysozyme release from rat neutrophils. Similar results were obtained by Finkelstein and coworkers (1977 and 1982) using human neutrophils. In order to determine whether the inhibitory effects of AUR on neutrophil phagocytosis and

degranulation were independent of one another, degranulation elicited by non-phagocytic, soluble stimuli was studied. AUR was shown to have a biphasic influence on the enzyme release evoked by the chemotactic tripeptide n-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe), (Hafström et al., 1983b). At concentrations less than 1 µM, AUR-treated cells released more lysozyme than did control cells. However, at concentrations greater than this, a significant inhibitory effect on both lysozyme and ß-glucuronidase release was observed. Studies utilizing other soluble stimuli have supported this observation (Coates et al., 1983; Hafström et al., 1984b). The calcium ionophore A23187 has been shown to elicit degranulation of azurophilic and/or specific granules (Goldstein et al., 1974; Goldstein et al., 1975a), but to be insensitive to the inhibitory actions of AUR (Hafström et al., 1983b). Wolach and coworkers (1982) demonstrated that AUR inhibited the release of granule components other than lysozyme and ß-glucuronidase. Release of myeloperoxidase and lactoferrin were prevented in AUR-pretreated cells. Marked inhibition of elastase (Kühn et al., 1985) and collagenase (Wojtecka-Lukasik et al., 1986) release were also noted. In all studies which involved GST, a failure to inhibit neutrophil degranulation was observed (Carevic, 1985). In general,

AUR inhibited neutrophil degranulation independently of phagocytosis and GST lacked this ability.

Another neutrophil effector function altered by AUR is the respiratory burst. The results of various studies parallel the results obtained with cellular degranulation; low concentrations of AUR elicit an enhanced respiratory burst which becomes significantly reduced at higher doses. The same results were obtained whether the cellular stimulus induced phagocytosis (Hafström et al., 1983a; Davis et al., 1983; Roisman et al., 1983), or was independent of phagocytosis, for example fMet-Leu-Phe (Davis et al., 1983; Coates et al., 1983), concanavalin A (Hafström et al., 1983a), and a tumour-promoting phorbol ester (Hafström et al., 1983b; Sung et al., 1984). In fact, the phorbol ester-induced respiratory burst was most sensitive to Au(I) complexes containing PEt_3-like ligands. Again, GST demonstrated poor inhibitory activity toward the respiratory burst.

In analysing the multiple activities of AUR (or gold compounds in general) in neutrophils, questions arise as to the nature of the inhibition observed: How do these compounds manifest their <u>in vitro</u> inhibitory activities? Do they affect each cellular response individually, or do they alter the transduction of inflammatory stimuli which results in overall suppression? To provide a background for these queries, the following

section reviews the current state of knowledge regarding signal transduction pathways in the neutrophil.

II SIGNAL TRANSDUCTION IN THE NEUTROPHIL

A. MEMBRANE RECEPTORS FOR CHEMOATTRACTANTS

The interaction of a stimulant with the neutrophil surface initiates oxidative metabolism of glucose, O_2 consumption, O_2^- generation (the respiratory burst), degranulation, changes in cellular shape and adherence capacities, chemotaxis, phagocytosis, and the generation of arachidonic acid metabolites. These events require the precise regulation of several signal transduction pathways. The series of events which lead to the induction of neutrophil responsiveness are incipient on the binding of cellular stimuli to specific plasma membrane receptors. Receptors have been identified for the peptide chemoattractants, such as fMet-Leu-Phe, a synthetic analogue of natural bacterial products (Becker, 1979); C5a, a cleavage product of the fifth component of complement, (Chenoweth and Hugli, 1978); crystal-induced chemotactic factor (Spilberg and Mehta, 1979); and the lipid chemoattractants, leukotriene B_4 (Goldman and Goetzyl, 1982) and platelet activating factor (Hwang et al., 1983). Other agents mimic the effects of physiological ligands and have become

valuable tools for the study of signal transduction. Some of these agents exhibit a similar type of receptor-mediated interaction with the neutrophil plasma membrane as those mentioned above (lectins, antibodies directed against cell surface antigens), whereas others act by perturbing the lipid phase of the plasma membrane (surfactants, phospholipases), and still others circumvent some of the initial steps in transmembrane signalling [calcium ionophores, phorbol esters, fluoride anion (F^-)]. Most of these cellular stimuli can activate more than one neutrophil response implying that a ligand-plasma membrane interaction generates a signal to the cell which is intracellularly amplified to reach various targets.

In a wide variety of cell types, changes in the plasmalemmal transmembrane potential are an initial step in stimulus-response coupling. When membrane potential changes were monitored in neutrophils upon activation with fMet-Leu-Phe, using either a labelled lipophilic cation [^{3}H]tetraphenyl phosphonium ion (Korchak et al., 1982), or the fluorescent carbocyanine dye, 3,3'-dihexyloxacarbocyanine iodide (Seligmann et al., 1980; Whitin et al., 1980), a depolarization event was observed. However, it was later determined that the carbocyanine dye was located primarily with the mitochondria and that the fluorescence loss associated

with a depolarization event was inhibited by cyanide, an electron transport inhibitor. Also, the loss of fluorescence was associated with a lag of approximately 3 s. Therefore, the depolarization following cellular activation by fMet-Leu-Phe is not immediate and unlikely to be an initiating event in the activation sequence. Hyperpolarization of neutrophil membranes by the ionophores valinomycin and nigericin, and depolarization by high extracellular K^+, do not stimulate cellular O_2 consumption (Romeo et al., 1975; Mottola and Romeo, 1982). As well, a tumour-promoting phorbol ester was shown to produce a large membrane depolarization without possessing chemotactic activity toward neutrophils (Mottola and Romeo, 1982), thus demonstrating that these changes in membrane potential do not play a major role in the transduction of external stimuli to modified neutrophil behaviour.

The receptor for formylated peptides has been studied extensively. It has an estimated molecular weight between 55,000 and 70,000 (Niedel et al., 1980; Painter et al., 1982; Allen et al., 1986), and exists in two affinity states; a high-affinity state (K_D=0.5 nM) representing approximately 25% of the total receptor population, and a low affinity state (K_D=24 nM), (Koo et al., 1982; Mackin and Becker, 1982). There are approximately 55,000 fMet-Leu-Phe binding sites present

per cell (Koo et al., 1982), and it has been postulated that an intracellular pool of receptors is recruited to the plasma membrane during chemoattractant-induced degranulation (Fletcher and Gallin, 1983). Following ligand binding, the receptor partakes of receptor-mediated endocytosis (Sklar et al., 1984). Receptors complexed in a high-affinity form have been shown to form rapid association with the contractile elements (cytoskeletal system) in the neutrophil cytoplasm (Painter et al., 1984; Jesaitis et al., 1984; Jesaitis et al., 1985). This type of association has also been demonstrated with leukotriene B_4 receptors (Naccache et al., 1984). Since these events precede internalization, it is suggested that the association between the ligand-receptor complex and the cytoskeleton may be necessary for receptor-mediated endocytosis as well as other cellular events involved in cellular shape and movement.

B. THE INVOLVEMENT OF GUANINE NUCLEOTIDE-BINDING REGULATORY PROTEINS

Guanine nucleotide-binding regulatory proteins (G-proteins) have been implicated in a number of receptor transduction systems including the adenylate cyclase system (Gilman, 1984) and photoreceptors (Fung et al., 1981). Of the G-proteins known, all are

heterotrimers composed of alpha, beta, and gamma subunits. The alpha subunits contain binding sites for guanine nucleotides and F^- and most are substrates for various bacterial toxins which catalyze their adenosine diphosphate-ribosylation. G-proteins represent a family of homologous regulatory proteins whose function is to serve as transducers of information from receptors to cellular effector systems.

Neutrophils stimulated to phagocytose by the introduction of starch particles, were shown to have an increased metabolic turnover of inositol lipids (Karnovsky and Wallach, 1961; Sastry and Hokin, 1966). Using fMet-Leu-Phe as a cellular stimulus, neutrophils were shown to incorporate increased amounts of ^{32}P into phosphatidylinositol which accompanied activation of the respiratory burst, degranulation and calcium fluxes (Bennet et al., 1980). The hypothesis that phospholipase C-mediated hydrolysis of polyphosphoinositides is an early event in signal tranduction (see next section), and that G-proteins couple the fMet-Leu-Phe receptor to phospholipase C activation, has been tested by several groups (Volpi et al., 1983; Yano et al., 1983; Dougherty et al., 1984; Bradford and Rubin, 1985). It has been demonstrated that conversion of the high-affinity form of the fMet-Leu-Phe receptor to the low affinity form is regulated by guanine

nucleotides suggesting the involvement of a G-protein in cellular activation through the fMet-Leu-Phe receptor (Koo et al., 1983; Snyderman et al., 1984; Lad et al., 1985a). In 1984, Bokoch and Gilman demonstrated a direct association of a G-protein with occupancy of the fMet-Leu-Phe receptor by utilizing pertussis toxin as a G-protein probe of intact cells. Treatment of cells with pertussis toxin, which catalytically inactivates certain G-proteins, Gi (Hildebrandt et al., 1983), resulted in the attenuation of fMet-Leu-Phe-stimulated degranulation, arachidonic acid metabolism and the respiratory burst. Subsequent studies with the toxin showed inhibition of chemotaxis (Becker et al., 1985), actin polymerization (Shefcyk et al., 1985), and phagocytosis (Verghese et al., 1985). When direct measurements of fMet-Leu-Phe receptor-stimulated phosphatidylinositol breakdown were made, it was observed that pertussis toxin markedly inhibited this process (Brandt et al., 1985; Smith et al., 1985; Volpi et al., 1985). Using membrane preparations from neutrophils and other cells in which an activatable phospholipase C was present, Smith and coworkers (1985) demonstrated significant breakdown of phosphatidyl-inositol 4,5-bisphosphate by fMet-Leu-Phe only in the presence of a guanine nucleotide. This breakdown was totally blocked in membranes obtained from cells which

had previously been treated with pertussis toxin. Cockcroft and Gomperts (1985) demonstrated that phosphatidylinositol hydrolysis and inositol phosphate production in neutrophil plamsa membranes could be stimulated by guanine nucleotides and displayed sensitivity to pertussis toxin. Taken together, these findings provide evidence for the involvement of a G-protein, similar to Gi of the adenylate cyclase system, in coupling surface receptors to phospholipase C activation in the neutrophil.

Neutrophil activation by other chemoattractants such as C5a (Becker et al., 1985), leukotriene B_4 (Goldman et al., 1985), and platelet activating factor (Lad et al., 1985b; Naccache et al., 1985), have been shown to be sensitive to pertussis toxin suggesting that receptors for these stimuli may also couple to phospholipase C through the pertussis toxin-sensitive G-protein in a manner similar to fMet-Leu-Phe receptors.

C. INOSITOL PHOSPHATES AND CALCIUM

Phospholipase C (1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase [EC 3.1.4.11]), catalyzes the hydrolysis of a specific class of membrane phospholipids, the polyphosphatidylinositols, thereby generating two intracellular second messengers, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. Represen-

tative of a minor component of the total membrane phospholipids (Berridge, 1984; Williamson et al., 1985), the phosphatidylinositides exist in three forms; phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate, in a ratio of approximately 90:5:5. They appear in the inner leaflet of the plasma membrane in all eukaryotic cells where they become susceptible to hydrolytic cleavage by phospholipase C. This enzyme separates the inositol phosphates from the glycerol backbone to form 1,2-diacylglycerol and either inositol 1-phosphate, inositol 1,2-cyclic phosphate, inositol 1,4-bisphosphate or inositol 1,4,5-trisphosphate (Putney, 1982; Berridge, 1984; Berridge and Irvine, 1984, Hokin, 1985; Williamson et al., 1985; Michell, 1986). Metabolism of 1,2-diacylglycerol, by a diglyceride lipase, leads to the formation of a monoacylglycerol and a fatty acid, usually arachidonic acid. Alternatively, 1,2-diacylglycerol can be phosphorylated to phosphatidic acid by a diglyceride kinase. Phosphatidic acid is the precusor for many phospholipids including the phosphatidylinositols. An additional pathway of phosphatidic acid metabolism is its hydrolysis to lysophosphatidic acid and arachidonic acid by a specific phospholipase A_2. The latter can give rise to eicosanoids such as prostaglandins, thromboxanes, prostacyclin and

leukotrienes, all potent mediators of inflammation. Phospholipase A_2 is a calcium-dependent enzyme which hydrolyzes other phospholipids, most notably, phosphatidylcholine, providing another source of arachidonic acid (Feinstein and Sha'afi, 1983; Decker, 1985). Figure 3 illustrates the pathways of polyphosphoinositide metabolism.

As mentioned in the previous section, several receptor systems are able to stimulate the turnover of membrane inositol phospholipids. Such systems have also been shown to mobilize intracellular calcium in response to ligand binding (Michell, 1975; Berridge, 1984). Observations from studies performed with permeabilized cell preparations have led to the proposal that the formation of inositol 1,4,5-trisphosphate, resulting from the receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate, is the primary mechanism for intracellular calcium mobilization. In neutrophil systems permeabilized to allow inositol 1,4,5-trisphosphate access to intracellular calcium stores, this metabolite initiated calcium release from nonmitochondrial stores at concentrations expected from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (0.1 - 1.5 μM) (Prentki et al., 1984a). Verification of this result has been obtained in other cell types including Swiss 3T3 cells (Irvine et al., 1984), peritoneal

Figure 3: The metabolism of inositol phospholipids and diacylglycerol.

PtdIns - phosphatidylinositol

PtdIns(4)P - phosphatidylinositol 4-phosphate

PtdIns(4,5)P_2 - phosphatidylinositol 4,5-bisphosphate

PLC - phospholipase C

DAG - 1,2-diacylglycerol

Ins(1,4,5)P_3 - inositol 1,4,5-trisphosphate

Ins(1,4)P_2 - inositol 1,4-bisphosphate

Ins(1)P - inositol 1-phosphate

Ins - inositol

PA - phosphatidic acid

PKC - protein kinase C

ER - endoplasmic reticulum

ATP - adenosine triphosphate

CTP - cytidine triphosphate

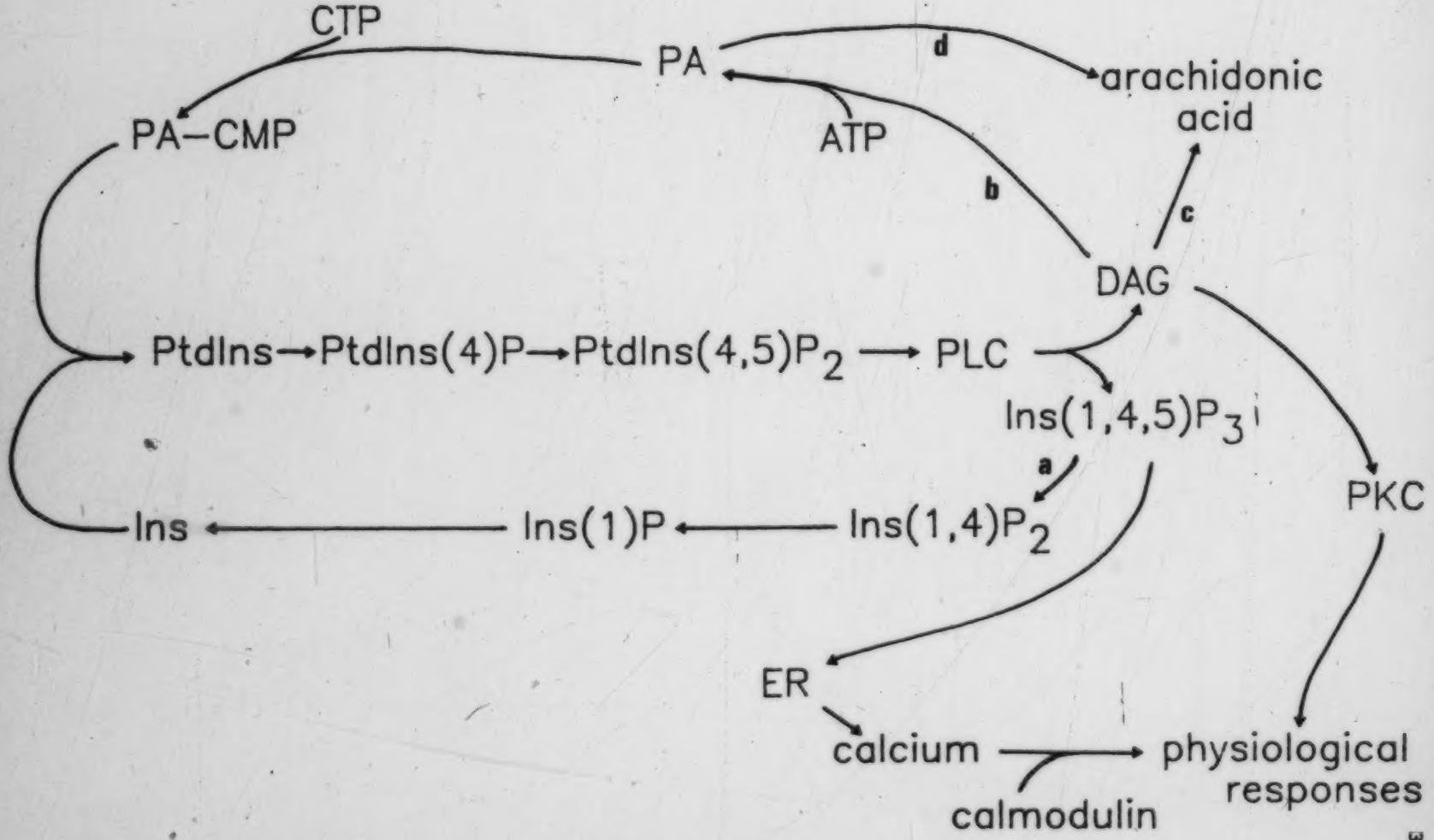
CMP - cytidine monophosphate

a - inositol trisphosphate 5'-phosphomonoesterase

b - diglyceride kinase

c - diglyceride lipase

d - phospholipase A_2


CTP
PA
d
arachidonic acid
PA–CMP
ATP
b
c
DAG
PtdIns→PtdIns(4)P→PtdIns(4,5)P2
PLC
Ins(1,4,5)P3
a
PKC
Ins
Ins(1)P
Ins(1,4)P2
ER
calcium
physiological responses
calmodulin

macrophages (Hirata et al., 1984), and hepatocytes (Burgess et al., 1984). In platelets and photoreceptor cells, inositol 1,2-cyclic phosphate was shown to cause a release of calcium from intracellular stores (Wilson et al., 1985). Thus, in addition to inositol 1,4,5-trisphosphate, this cyclic metabolite may also contribute to the mobilization of intracellular calcium.

Localization of the stores of intracellular calcium has been achieved through studies on subcellular fractions of hepatocytes (Dawson and Irvine, 1984; Spät et al., 1986), platelets (O'Rourke et al., 1985), neutrophils (Spät et al., 1986), and insulinoma cells (Prentki et al, 1984b). All studies indicate that inositol 1,4,5-trisphosphate-stimulated calcium release is originating from the smooth endoplamsic reticulum.

An important aspect concerning the function of inositol 1,4,5-trisphosphate as a second messenger is that of timing: Its production by the stimulated cell must precede the mobilization of intracellular calcium and its presence must be transient, that is, its production must be halted soon after it is produced. Inositol 1,4,5-trisphosphate formation by stimulated Calliphora salivary glands (Berridge, 1983; Berridge et al., 1984), hepatocytes (Thomas et al., 1984), and GH_3 cells (Drummond et al., 1985), was immediate and preceded the physiological responses of these cells.

Inositol 1,4,5-trisphosphate 5'-phosphomonoesterase (Downes et al., 1982) is responsible for the attenuation of the second messenger activity of inositol 1,4,5-trisphosphate. It removes phosphate from the 5-position to produce inositol 1,4-bisphosphate, which lacks the ability to mobilize intracellular calcium. Other enzymes remove the phosphates from inositol 1,4-bisphosphate to regenerate inositol and subsequently, formation of the phosphoinositides (Berridge and Irvine, 1984). Thus, the transient appearance of inositol 1,4,5-trisphosphate is sufficient for it to function as a second messenger.

The maintenance of calcium homeostasis by cells is a remarkable achievement due to the 5,000 to 10,000-fold calcium concentration gradient across the plasma membrane. The cytosolic calcium concentration in resting cells ranges from 100 to 200 nM, whereas, in the extracellular fluid, concentrations are normally in the millimolar range. After cellular stimulation, cytosolic calcium levels rise to micromolar levels, however, homeostasis is maintained by a low natural permeability of the plasma membrane to calcium and by at least two adenosine triphosphate-dependent mechanisms by which calcium is pumped out of the cell against the gradient: (1) a calcium transport adenosine triphosphatase or calcium pump, and (2) a sodium-calcium exchanger, driven

by the sodium gradient, which is maintained by sodium-potassium adenosine triphosphatase (Blaustein, 1974; Carafoli and Zurini, 1982; Schatzman, 1985). Additionally, calcium released into the cell cytosol during activation may re-enter its internal storage site.

Calcium has been shown to be involved in the regulation of numerous physiological and biochemical processes of the cell (Rasmussen, 1970; Berridge, 1975; Rasmussen and Goodman, 1977). Its roles in cellular regulation are mediated by its interaction with various intracellular calcium-binding proteins such as troponin C, S-100 protein, parvalbumin, calmodulin and several protein kinases (Kretsinger, 1980). Ubiquitous to all eukaryotic cells, calmodulin is the major mediator of calcium action (Kakiuchi and Yamazaki, 1970). This protein shows high affinities toward calcium (Teo and Wang, 1973), and can undergo calcium-dependent associations with, and regulation of, numerous enzymes (Teshima and Kakiuchi, 1974; Lin et al., 1975). Calmodulin has been demonstrated to regulate enzymes involved in the metabolism of glycogen, calcium, cyclic nucleotides as well as contractile processes, many of which involve calmodulin-stimulated protein kinases and phosphatases. Free, inactive calmodulin is converted to an active conformation by the binding of calcium. A total of four calcium ions bind to calmodulin with

affinities ranging from 10^{-6} to 10^{-4} M, which then associates with various target enzymes and modulates their functioning (Wang et al., 1975). The binding of calcium results in a 10 to 100 thousand-fold increase in the affinity of calmodulin for its target enzymes. A schematic representation of calmodulin regulatory processes is presented in Figure 4.

D. DIACYLGLYCEROL AND PROTEIN KINASE C

Almost coincident with the discovery by Berridge (1983) that inositol 1,4,5-trisphosphate, an early product of phosphatidylinositol 4,5-bisphosphate hydrolysis, serves as a mediator of calcium mobilization from an intracellular store, was the evidence of Nishizuka and his colleagues concerning 1,2-diacylglycerol, the other product of this pathway, as an activator of a special kinase, protein kinase C (Takai et al., 1979a; Kishimoto et al., 1980; Mori et al., 1980; Kawahara et al., 1980; Sano et al., 1983). His group also postulated that the signal pathway through protein kinase C-mediated protein phosphorylation was separate from, and often synergistic to, the inositol 1,4,5-trisphosphate-mediated calcium signalling pathway in controlling various cellular functions.

Protein kinase C was first discovered by Nishizuka

Figure 4: Calmodulin regulatory pathways.

$[Ca^{2+}]_i$ - intracellular calcium concentration

CaM - calmodulin

(Wang et al., 1985)

Page 37 has been removed because of the inavailability of copyright permission. The material included a pathway of protein regulation via calcium and calmodulin taken from Wang, J.H., Pallen, C.J., Sharma, R.K, Adachi, A.-M., Adachi, K. Cur. Top. Cell Reg. 27, 419, 1985.

and coworkers in 1977 (Inoue et al., 1977). It is widely distributed in animals (Kuo et al., 1980), and occurs in many tissues and organs within the same organism. It consists of one polypeptide with a relative molecular weight of approximately 77,000. Protein kinase C is a serine and threonine protein kinase which requires calcium and phospholipid (primarily phosphatidylserine), for its activation. The binding of 1,2-diacylglycerol dramatically increases the affinity of this enzyme for calcium ($<10^{-7}$ M), rendering it fully active without a net increase in cellular calcium concentration (Kishimoto et al., 1980; Kaibuchi et al., 1981).

When tested in vitro, protein kinase C displays broad substrate specificity (see Table II). It phosphorylates proteins at serine and threonine residues with particular preference for those hydroxy amino acids located at the amino terminal end of the polypeptide (Krebs and Beavo, 1979). As well, basic amino acid residues flank the amino and carboxyl ends of the seryl and threonyl sites of the target protein (Kishimoto et al., 1985). The enzyme can undergo autophosphorylation in the presence of calcium, phospholipid and diacylglycerol, although the significance of this reaction is unknown at present.

Protein kinase C can be activated by limited

Table II: Various substrates of protein kinase C.

Receptor proteins	Enzymes
EGF receptor	glycogen phosphorylase kinase
insulin receptor	glycogen synthase
somatomedin C receptor	phosphofructokinase
transferrin receptor	HMG CoA reductase
IL2 receptor	tyrosine hydroxylase
ß-adrenergic receptor	NADPH oxidase
IgE receptor	cytochrome P450
	guanylate cyclase
Membrane proteins	DNA methylase
Ca^{2+}-transport ATPase	myosin light chain kinase
Na^+/K^+ ATPase	initiator factor 2
Na^+ channel protein	
glucose transporter	**Other proteins**
G-protein	fibrinogen
HLA antigen	retinoid-binding protein
	vitamin D-binding protein
Cytoskeletal proteins	ribosomal S6 protein
myosin light chain	GABA modulin
troponin T and I	stress proteins
vinculin	myelin basic protein
filamin	high-mobility group proteins
caldesmon	middle T antigen
microtubule proteins	$pp60^{src}$ protein

proteolysis with calcium-dependent thiol proteases (calpain), into two functionally distinct domains: (1) a hydrophilic, 50 kD, functional, catalytic domain (Inoue et al., 1977; Takai et al., 1977; Kishimoto et al., 1983a; Tapley and Murray, 1985; Melloni et al., 1986), and (2) a hydrophobic, 32 kD, polypeptide regulatory domain containing the calcium, phospholipid and diacylglycerol binding sites (Lee and Bell, 1986). The 50 kD catalytic domain retains the ability to function as a protein kinase, however, it has lost its calcium and phospholipid dependencies. These serine proteases, which are activated at 1 to 10 μM calcium, preferentially cleave membrane-bound protein kinase C, although, the physiological significance of this reaction is currently under investigation (Tapley and Murray, 1984; Melloni et al., 1985; Melloni et al., 1986).

In most cells, under normal conditions, protein kinase C is recovered mainly from the soluble fraction. However, upon cellular activation, this enzyme is recovered in the membrane fraction of the cell, a process often referred to as translocation (Kraft and Anderson, 1983; Hirota et al., 1985; Wolf et al., 1985a; Wolf et al., 1985b). It remains uncertain as to how this redistribution process occurs. One suggestion is that diacylglycerol mediates the translocation from a

soluble, inactive, cytosolic enzyme to an activated, membrane-associated form. Another hypothesis suggests that inactive protein kinase C exists in a state of loose association with the plasma membrane. When activated, the transient appearance of diacylglycerol somehow intensifies the kinase's association with the membrane (Kraft and Anderson, 1983). This theory has been substantiated by the studies of Helfman and coworkers (1983) who reported that the apparent cytosolic localization of protein kinase C in unstimulated cells was an artifact resulting from calcium chelation by the EDTA-containing buffer used during cellular homogenization. In the absence of chelators during cell disruption, this group found that protein kinase C expressed a weak affinity for the membrane fraction characteristic of a plasma membrane associated protein.

A highly specific lipid-protein interaction is required for complete protein kinase C activation. Mono- and triacylglycerols have been shown to lack stimulatory activity toward protein kinase C (Kishimoto et al., 1980). Diacylglycerols of the 1,2 configuration possess the capacity to activate protein kinase C. They contain at least one unsaturated fatty acid, commonly arachidonic acid, at position 2 (Mori et al., 1982). Studies of Rando and Young (1984) have demonstrated that

2,3- and 1,3-diacylglycerols are completely ineffectual as protein kinase C activators. It has been postulated that approximately one molecule of diacylglycerol has the potential to activate protein kinase C in the presence of between 4 and 10 molecules of phosphatidylserine (Hannun et al., 1985; Ganong et al., 1986).

Transiency was demonstrated for the stimulated production of inositol 1,4,5-trisphosphate. Analogously, 1,2-diacylglycerol formation and appearance in cell membranes is also transient. Within a very short time after its formation, this lipid is rapidly metabolized (Kikkawa et al., 1983; Sano et al., 1983; Fisher, et al., 1984; Majerus et al., 1985; Downes and Michell, 1985), due to both its phosphorylation to phosphatidic acid and subsequent channelling back to inositol phospholipids or to its degradation by diglyceride lipase to arachidonic acid (see Figure 3). Although protein kinase C is active for brief periods of time, the results of enzyme activation may persist depending upon the stability of the phosphate covalently attached to each substrate.

Diacylglycerols, such as diolein, cannot be readily incorporated into the plasma membrane when added exogenously to cells. Synthetic diacylglycerols have been prepared which readily intercalate into the cell membrane and activate the kinase directly in the absence

of phosphoinositide turnover. Examples of these are 1-oleoyl-2-acetylglycerol (Nishizuka, 1984a; Nishizuka, 1984b), 1,2-didecanoylglycerol, and 1,2-dioctanoylglycerol (Lapetina et al., 1985). These compounds have been utilized experimentally to explore protein kinase C activity. Structurally similar to diacylglycerol are the phorbol ester tumour promotors, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) being a prototype. These compounds were first isolated from the oil of the seed Crotum tiglium and have since been found to be potent activators of protein kinase C in vitro as well as in vivo (Castagna et al., 1982; Yamanishi et al., 1983). Phorbol esters are hydrophobic agents which resemble diacylglycerol by virtue of their acyl moieties on positions 12 and 13 of their third ring (see Figure 5). Tumour promoting agents structurally unrelated to the phorbol esters have also been shown to activate protein kinase C. Examples include aplysia toxin, debromoaplysia toxin, teleocidin, and mezerein (Fujiki et al., 1984; Miyake et al., 1984; Couturier et al., 1984). It has been proposed that these agents alter membrane properties which results in activation of the kinase.

Like diacylglycerol, phorbol esters increase the affinity of protein kinase C for calcium, resulting in full activation of the enzyme at physiological calcium

Figure 5: Structures of synthetic diacylglycerol and a tumour-promoting phorbol ester.

TPA contains a diacylglycerol-like structure as shown in the dotted figure.
(Nishizuka, 1984a)

Page 45 has been removed because of the inavailability of copyright permission. The material included the molecular structures of 1-oleoyl-2-acetyl glycerol and 12-O-tetradecanoyl-phorbol-13-acetate taken from Nishizuka, Y. Nature (London) 308, 693, 1984.

concentrations. Thus, phorbol esters capable of tumour promotion are able to mimic the effect of diacylglycerol in activating protein kinase C (Castagna et al., 1982). In addition, it has been shown that protein kinase C functions as the endogenous receptor protein for tumour promotors (Niedel et al., 1983; Nishihira and O'Flaherty, 1985; König et al., 1985; Jeng et al., 1986). However, since phorbol esters cannot rapidly be degraded by the endogenous enzymes which inactivate diacylglycerol, prolongation of cellular responsiveness results upon exposure to these agents. This property may be, in part, responsible for their hyperplasiogenic activities.

Recently, Parker and coworkers (1986) elucidated the complete amino acid sequence of protein kinase C using sequence analysis and recombinant DNA technology. Antibodies to specific regions of the polypeptide were prepared to probe the domain structure predicted for the kinase and to provide clues to the structure and function of this protein. An interesting feature, present at the amino terminus, is a tandem repeat containing six cysteine residues. Each repeat possesses the sequence Cys-X_2-Cys-X_{13}-Cys-X_2-Cys, a pattern observed for various other classes of proteins (Berg, 1986). These include certain cytochromes c, iron-sulfur proteins, small disulfide-rich proteins such as toxins,

hormones and protease inhibitors, high-sulfur keratins, metallothioneins, and proteins implicated in nucleic acid binding. The cysteine-rich region of protein kinase C has generated much interest in that it has the potential to interact with DNA as well as possessing characteristics of a metalloprotein.

Preceding the catalytic domain of the protein is a stretch of approximately 200 amino acids. The only calcium-binding site which bears structural resemblance to the binding domain in other calcium-binding proteins such as calmodulin (van Eldik et al., 1982), is located here. The catalytic moiety, demonstrated to be proteolytically activated in vitro by limited trypsinolysis (Inoue et al., 1977), was found to have a domain limit between 43 and 47 kD. This correlated well with the 43 to 50 kD protein estimated by gel filtration chromatography. This catalytic domain was found to be structurally homologous to other serine, threonine and tyrosine protein kinases.

Based on trypsinization studies and the known requirements for full protein kinase C activity, a model was predicted for the activation of this enzyme (Figure 6). The hinge region located between the regulatory and catalytic domains, is the site of proteolytic cleavage. In this model, a single regulatory domain maintains a catalytic domain in an inactive state until ligand is

Figure 6: Model for protein kinase C activation.

DG - diacylglycerol

P-lipid - phospholipid

Sub - substrate binding site

ATP - adenosine triphosphate

Ca^{2+} - calcium

CANP - calpain

(Parker et al., 1986)

Page 49 has been removed because of the inavailability of copyright permission. The material included a model for protein kinase C activation taken from Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D., Ullrich, A. Science 233, 853, 1986.

bound. This type of structure has also been identified for the cyclic guanosine monophosphate-dependent protein kinase, where it has been predicted that this enzyme was derived from the fusion of two genes; one coding for the regulatory domain, and the other for the catalytic domain.

The work of Coussens and coworkers (1986) has changed the popular perception of protein kinase C as a single protein involved in cellular regulation and proliferation. Using in situ hybridization and Southern analysis, they have localized a family of three genes, coding for alpha, beta, and gamma forms of protein kinase C, to distinct chromosomes in the bovine, human and rat genome. These genes encode for polypeptides that are indistinguishable by physical and functional analyses. Thus, the diversity of cellular responses observed in various studies may be due to the activation of one or more of these polypeptides.

E. COORDINATED REGULATION OF CELLULAR FUNCTIONS

Receptor-mediated polyphosphoinositide breakdown initiates two responses in the cell; the generation of a calcium-releasing inositol 1,4,5-trisphosphate, and a protein kinase C-activating diacylglycerol. Studies demonstrating the independent induction of calcium mobilization and protein kinase C activation have

employed the exogenous addition of a calcium ionophore and either a synthetic diacylglycerol or a phorbol ester tumour promotor, respectively. Initial studies of this type were performed on platelets since the bifurcating pathways can be easily monitored by measuring the phosphorylation of either a 40 or 20 kD protein. Platelets respond to thrombin, platelet activating factor, collagen and other stimuli, by secreting lysosomal enzymes, serotonin, ADP, growth factors, and various other constituents. When thrombin binds to its cell surface receptor on the platelet, there is a prompt hydrolysis of phosphatidylinositol 4,5-bisphosphate resulting in the production of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, followed by increases in the levels of phosphatidic acid and arachidonic acid (Rink et al., 1985; Feinstein et al., 1985). Consequently, there is a transient increase in intracellular calcium concentration which results from both the release from an intracellular pool and an increase in the rate of calcium influx from the extracellular fluid (Rink et al., 1985). The subsequent activation of calmodulin-dependent protein kinases, myosin light chain kinase and protein kinase C, results in the concomitant phosphorylation of specific protein substrates, namely, myosin light chain (a 20 kD protein), and a 40 kD protein of unknown origin (Lyons

et al., 1975; Haslam and Lynham, 1977; Kawahara et al., 1980; Ieyasu et al., 1982; Kaibuchi et al., 1983; Sano et al., 1983). Activation of protein kinase C directly promotes the phosphorylation of the 40 kD substrate exclusively, whereas, the calcium ionophore, A23187, promotes phosphorylation of the 20 kD substrate. It has been shown that protein kinase C activation and calcium mobilization synergistically elicit platelet responses of serotonin and lysosomal enzyme release as well as phosphorylation of both protein substrates (Kaibuchi et al., 1982; Kaibuchi et al., 1983; Yamanishi et al., 1983; Kajikawa et al., 1983). This type of synergism has also been noted in many other cell systems involving immediate responses similar to those of the platelet (see Table III), and such long-term responses as cellular proliferation and gene expression (Mastro and Mueller, 1974; Watanabe et al., 1981; Wilkinson and Morris, 1983; Zatz, 1985; Otani et al., 1985; Jetten et al., 1985; Johnson et al., 1985; Degen et al., 1985; de Bustros et al., 1985; Murdoch et al., 1985; Siebert and Fukuda, 1985).

The neutrophil is an interesting and complicated cell in which to study the coordinated involvement of protein kinase C and calcium in the regulation of its responses. Studies focusing on activation of the respiratory burst have furnished both positive and

Page 53 has been removed because of the inavailability of copyright permission. The material included a table listing the immediate cellular responses under dual regulation by protein kinase C and calcium taken from Takai, Y., Kaibuchi, K., Tsuda, T., Hoshijima, M. J. Cell. Biochem. 29, 143, 1985.

negative evidence for the involvement of either one or both of these regulatory components. FMet-Leu-Phe, leukotriene B_4, concanavalin A, platelet activating factor, A23187 and opsonized zymosan, have all been shown to activate the neutrophil respiratory burst subsequent to an initial rise in intracellular calcium concentration (Naccache et al., 1979; Hoffstein, 1979; Mottola and Romeo, 1982; Pozzan et al., 1983; White et al., 1983; Fujita et al., 1984; Korchak et al., 1984; Lew et al., 1984a; Volpi et al., 1984; Gennaro et al., 1984; Ohta et al., 1985; Lew et al., 1985; Lad et al., 1985c; Sawyer et al., 1985). Neutrophils depleted of calcium could not elicit a respiratory burst upon exposure to these agents (Lew et al., 1984b; Grzeskowiak et al., 1986). Other studies have provided evidence against an involvement of calcium in the activation of the respiratory burst. Ionomycin, a calcium ionophore, induces a similar increase in intracellular calcium concentration to that of fMet-Leu-Phe, yet it is unable to activate the respiratory burst (Pozzan et al., 1983). The respiratory burst can be induced by direct activators of protein kinase C in normal and calcium-depeleted neutrophils (DiVirgilio et al., 1984; Grzeskowiak et al., 1986). This has been shown to be associated with an increase in protein phosphorylation and shows sensitivity to various inhibitors of protein

kinase C (Halfman et al., 1983; White et al., 1984; Fujita et al., 1984; Cooke and Hallett, 1985; Gerard et al., 1986). Translocation of protein kinase C from the neutrophil cytosol to the plasma membrane was also demonstrated when the respiratory burst was activated by TPA (Wolfson et al., 1985; Myers et al., 1985; Gennaro et al., 1986; Nishihira et al., 1986; Pontremoli et al., 1986). When calcium-depleted neutrophils were treated with a non-stimulatory dose of TPA, fMet-Leu-Phe was able to activate the respiratory burst (Grzeskowiak et al., 1986). This form of synergism was also evident using the synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol as a protein kinase C activator (Dewald et al., 1984). Studies of Bromberg and Pick (1984) and Heyneman and Vercauteren (1984), involving the activation of neutrophil NADPH oxidase in a cell-free system, indicated that a cytosolic factor was required for complete activation of the enzyme complex. This requirement was satisfied when the factor was substituted by purified protein kinase C (Bromberg and Pick, 1984; Heyneman and Vercauteren, 1984; Bromberg and Pick, 1985; McPhail et al., 1985; Curnette, 1985; Cox et al., 1985).

Although an increase in intracellular calcium concentration may be necessary for neutrophil degranulation and chemotaxis, its role in the activation

of the respiratory burst remains obscure. With some cellular stimuli, calcium may be required to elicit a response. For example, leukotriene B_4, concanavalin A, and platelet activating factor do not promote translocation of protein kinase C in their activation of the respiratory burst (Nishihira et al., 1986). In other situations, an increase in calcium concentration may not be sufficient to elicit this response without coexisting with other signals. On the other hand, evidence seems to favour the active involvement of protein kinase C, with or without a concomitant rise in intracellular calcium concentration, in the elicitation of the respiratory burst.

III OBJECTIVES

The underlying theme of this research project is to elucidate the mechanism(s) by which gold coordination complexes manifest their <u>in vitro</u> antiinflammatory effects. The neutrophil will be utilized as a model cell in which to study signal-response coupling and the alterations in this scheme produced by the presence of gold compounds.

Initial studies will focus on the physiological functions of the neutrophil stimulated by soluble agents, such as degranulation, arachidonate metabolism, and the respiratory burst. The products of these

cellular responses, hydrolytic enzymes, eicosanoids, and active oxygen species, respectively, are all potent mediators of inflammation. The gold compound AUR has previously been shown to suppress neutrophil degranulation stimulated by particulate and soluble agents (DiMartino and Walz, 1977; Finkelstein et al., 1977; Finkelstein et al., 1982; Hafström et al., 1983a; Hafström et al., 1983b; Coates et al., 1983; Hafström et al., 1984a). At similar concentrations, the generation of superoxide anions was suppressed by AUR (Hafström et al., 1983a; Davis et al., 1983; Coates et al., 1983; Roisman et al., 1983). An alteration in neutrophil de novo eicosanoid production by the gold compounds has not yet been investigated. To complement previous studies, these cell functions will be examined in an attempt to localize the action of the gold compound(s) to components of the signal transduction cascade.

The generation of superoxide anions by the neutrophil is elicited by agents such as fMet-Leu-Phe which bind to specific receptors on the plasma membrane, F^- which acts at the level of a G-protein, and phorbol esters which directly activate protein kinase C. Thus, by measuring the respiratory burst induced by each of these agents in cells treated with either AUR or GST, it may be possible to eliminate certain arms of the transduction scheme and focus on others which may be

targets of gold drug action.

Localization of gold drug action in the neutrophil will be probed further by investigating elements of the signal transduction sequence independently of one another. Inositol 1,4,5-trisphosphate production by the action of phospholipase C on phoshatidylinositol 4,5-bisphosphate, will be monitored indirectly by measuring changes in intracellular calcium levels induced by receptor-mediated stimuli, since this metabolite possess the ability to mobilize intracellular calcium stores. Protein kinase C activity is recovered primarily in the cytosol of resting cells. A direct effect of the gold drugs on the activity of this enzyme will be investigated. In addition, an alternate source of partially purified protein kinase C will be utilized to determine whether the effect of the drugs, if any, is cell specific. Since cellular activation can be identified by a redistribution of protein kinase C activity from the cytosolic to the particulate cellular fraction, it will be possible to monitor the effects of the gold compounds on this activation process. To overcome possible aberrations in enzyme activity measurements, the fractions will also be probed with polyclonal anti-protein kinase C antibodies to provide qualitative evidence of translocation.

The proposed structure of protein kinase C is a

single polypeptide composed of two domains, a regulatory domain and a catalytic domain. It is possible that if the gold compounds were found to alter enzymatic activity, that this could occur via interference with ligand binding (the regulatory domain), or alternatively, with its kinase activity (catalytic domain). To complement the enzyme assays, protein kinase C binding assays will also be performed using a phorbol ester ligand.

Subsequent to increased levels of intracellular calcium and activation of protein kinase C that arise in the activated cell is the phosphorylation of intracellular target proteins. Protein phosphorylation is mediated by calcium and calmodulin-dependent kinases, protein kinase C, cyclic adenosine monophosphate-dependent kinase , as well as other enzymes. These main phosphorylation pathways can be stimulated independently of one another making it possible to study each kinase function and its susceptibility to gold drugs.

CHAPTER TWO

MATERIALS AND METHODS

A. MATERIALS

The following reagents were purchased from Sigma Chemical Co., St. Louis, Missouri: Trizma base, histone type III-S, 1,2-diolein, L-alpha-phosphatidyl-L-serine, adenosine triphosphate, casein, NP-40, lactate dehydrogenase, trypan blue, NAD, NADPH, lysozyme, Micrococcus lysodeikticus, cytochalasin B, fMet-Leu-Phe, ß-glucuronidase, phenolphthalein glucuronide, arachidonic acid, fetal calf serum, ferricytochrome c type VI, xanthine, xanthine oxidase, BSA, PMSF, leupeptin, Triton X-100, dithiothreitol, polyethylene glycol 6000 and trichloroacetic acid.

Both HBSS and Hepes were purchased from Gibco Laboratories, Grand Island, New York.

Ficoll-Paque was obtained from Pharmacia Inc., Dorvall, Quebec.

The following reagents were purchased from Bio-Rad Laboratories, Richmond, California: SDS, acrylamide, bis-acrylamide, TEMED, bromphenol blue, Coomassie Blue R-250, SDS-PAGE low MWt standards, Zeta-probe membrane and glycine.

Human gamma-globulin was supplied by Connaught Laboratories Ltd., Willowdale, Ontario.

Nitrocellulose membranes, 0.45 µm, were purchased from Mandel Scientific Co., Rockwood, Ontario.

TPA, phorbol 12,13-dibutyrate, Quin 2, Quin 2/AM and Fura 2/AM were purchased from CalBiochem Brand Biochemicals, San Diego, California.

The Folin-Ciocalteu reagent, ammonium persulfate and urea were obtained from Fisher Scientific Co., Fairlawn, New York.

Carrier free [^{32}P] orthophosphate was supplied by ICN Biomedicals Canada Ltd., Montreal, Quebec. [γ-^{32}P]ATP was from Amersham Canada Ltd., Oakville, Ontario. [^{3}H]phorbol 12,13-dibutyrate, the [^{3}H]-leukotriene C_4 radioimmunoassay kit and [14,15-^{3}H]-leukotriene B_4 for use in the leukotriene B_4 radioimmunoassay, were purchased from NEN Dupont Canada, Inc., Lachine, Quebec.

Kodak XAR5 film was purchased from Eastman Kodak Co., Rochester, New York.

The antirheumatic compound, AUR, was a gift from Smith, Kline and French Laboratories, Philadelphia, Pennsylvania. GST was a gift from Poulenc, Montreal, Quebec.

The leukotriene B_4 standard and leukotriene B_4 antibody were purchased from Welmark Diagnostics Inc., Guelph, Ontario. The [^{3}H]prostaglandin E_2 radioimmunoassay kit was purchased from Seragen Inc., Boston, Massachusetts.

All remaining chemicals were reagent grade.

All buffers were prepared in deionized, distilled water filtered through a Millipore RQ Water Purifier. Most reagents were water-soluble and were prepared at the required concentrations either in water or in a suitable buffer. AUR was dissolved directly in ethanol and diluted in either water or buffer as indicated. TPA, phorbol 12,13-dibutryate, phosphatidylserine, diolein, cytochalasin B, arachidonic acid, Quin 2, Quin 2/AM, Fura 2/AM, PMSF and leupeptin were initially dissolved in Me_2SO and used at a final concentration of Me_2SO that did not interfere with cellular functioning (Wong and Chew, 1982).

^{125}I-protein A was prepared according to the method of Brown et al. (1979) by Dr. David B. Glass and Dr. Laura A. Uphouse, Atlanta, Georgia.

B. ISOLATION OF NEUTROPHILS

Venous blood was drawn from healthy human volunteers into heparinized tubes. After centrifugation at 400 x g for 10 min, the plasma was removed and the compacted blood cells were resuspended in Hanks' Balanced Salt Solution (HBSS, composition: 137 mM NaCl; 5.4 mM KCl; 4.2 mM $NaHCO_3$; 5.6 mM glucose; 1.6 mM $CaCl_2$; 0.5 mM $MgCl_2$; 0.4 mM $MgSO_4$; 0.4 mM KH_2PO_4; 0.3 mM Na_2HPO_4; pH 7). Polypropylene tubes were used in all subsequent stages of neutrophil isolation and

handling as these cells have a tendency to adhere to glass. The cell suspension was gently layered on Ficoll-Paque, a low viscosity, erythrocyte aggregating agent, and centrifuged for 30 min at 400 x g. Aggregated erythrocytes as well as neutrophils sedimented completely through the Ficoll-Paque. Due to their lower density, mononuclear cells were unable to penetrate the Ficoll-Paque and were found at the interface between this agent and the HBSS medium (Boyum, 1968). The erythrocyte-neutrophil pellet was resuspended in a hypotonic salt solution (0.15 M NH_4Cl; 0.01 M $NaHCO_3$; 0.1 mM Na_2EDTA; pH 7.2), to achieve lysis of the erythrocyte component. The remaining neutrophils were washed in HBSS and cell density was adjusted using a Coulter counter, Model ZBI high-speed electronic particle counter. On average, a cell yield of 1-5 x 10^6 cells/mL of whole blood was obtained using this procedure with >95% neutrophils as determined by microscopic inspection.

C. CELLULAR VIABILITY

Cellular viability was examined using both the trypan blue exclusion criteria and the lactate dehydrogenase (LDH) assay. Trypan blue exclusion criteria for cell death involved a 1:1 dilution of cells (10^7/mL) to trypan blue dye (0.5% in phosphate

buffered saline) placed on a Neubauer hemocytometer and a percentage of viable cells calculated. Dead cells appeared blue under the light microscope due to the uptake of dye.

LDH (EC 1.1.1.27) assays were performed as previously reported by Wacker et al. (1956). Lactate dehydrogenase is a cytosolic enzyme and its release into the extracellular medium can be viewed as a criterion for cellular cytotoxicity. This enzyme, in the presence of L-lactate, catalyzes the reduction of the diphosphopyridine nucleotide, ß-nicotinamide adenine dinucleotide (NAD), with the concomitant generation of pyruvate:

$$\text{L-lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{L-pyruvate} + \text{NADH} + \text{H}^+$$

The presence of LDH was indicated by the reduction of NAD which was monitored spectrophotometrically by an increase in optical density at 340 nm, with a slit width of 1 nm, against a blank containing 0.05 M NAD, pH 7.5. Reaction mixtures consisted of 0.75 mL sodium phosphate buffer (0.1 M Na_2HPO_4, pH 8.8); 0.5 mL sodium lactate (0.16 M, pH 7.0); 0.15 M NAD (0.05 M, pH 7.5), and 0.1 mL of 15,000 x g supernatant from cells (10^7/mL) under various experimental conditions. A measure of total LDH content in cells was obtained from

the 15,000 x g supernatant of a sonicated cell suspension (six, 10 s bursts at 50-70 Watts on a Braunsonic 1510 sonicator).

D. CELLULAR DEGRANULATION

Both the enzyme activities of lysozyme and ß-glucuronidase were measured in order to assess release from neutrophil specific and azurophilic granules. Lysozyme (EC 3.2.1.17) hydrolyzes the ß-1,4-glucosidic linkages in the mucopolysaccharide cell wall of many bacteria including Micrococcus lysodeikticus. Assays for lysozyme release from both neutrophil granules were conducted by monitoring the decrease in absorbance at 450 nm in a suspension of this target microorganism (Shugar, 1952). Reaction mixtures consisted of 1.5 mL of a suspension of Micrococcus lysodeikticus (0.16 mg/mL) in phosphate buffer (67 mM NaH_2PO_4, pH 6.7) and 0.5 mL of a 15,000 x g cell supernatant (10^7/mL). Absorbance changes over a 2 min time interval at 450 nm were determined and compared to the total release of lysozyme in a 0.5% Triton X-100 treated cell suspension. Stimulated controls consisted of supernatants from cells treated for 10 min with 5 µg/mL cytochalasin B followed by a 5 min incubation with 1 µM fMet-Leu-Phe. A decrease in absorbance of 0.001/min at 450 nm and 25°C was representative of one unit of

lysozyme activity. Samples were assayed in polystyrene cuvettes at a slit width of 1 nm against a distilled water blank in a Shimadzu UV-240 Spectrophotometer.

ß-glucuronidase (EC 3.2.1.31) hydrolyzes a variety of conjugated glucuronides and is found in the neutrophil azurophilic granules. Assays for ß-glucuronidase release were modified from Fishman and coworkers, (1948), wherein phenolphthalein glucuronide was used as a substrate. An aliquot of cell supernatant (10^7/mL, 15,000 x g) was incubated at 37°C for 18 h with the enzyme substrate, phenolphthalein glucuronate (0.01 M), in acetate buffer, pH 4.6. The reaction was terminated by the addition of ice cold glycine buffer (0.2 M, pH 10.4), at which time absorbance readings of the various samples were taken at 540 nm, with a 1 nm slit width, against a distilled water blank. One unit of ß-glucuronidase activity was defined as that amount of enzyme cleaving one micromole of phenolphthalein glucuronide per min at 37°C. The supernatant obtained from the stimulated cells (cytochalasin B + fMet-Leu-Phe) was used as a positive control (see above).

E. ASSAYS OF EICOSANOID SYNTHESIS

Upon the addition of arachidonic acid substrate to a suspension of neutrophils, the production of eicosanoids, prostaglandins and leukotrienes, can be

assayed through the stimulation of cyclooxygenase and lipoxygenase, respectively. Neutrophils were adjusted to 10^7 cells/mL in HBSS and subjected to varying test conditions. Arachidonic acid (0.01 mg/mL), fMet-Leu-Phe (1 µM) ± cytochalasin B (5 µg/mL) were added to the cell suspensions and the reaction was terminated at various time intervals (0.5 - 60 min) by centrifugation at 15,000 x g for 2 min. To minimize oxidative degradation of the metabolites, the cell supernatants were immediately pooled and stored at -70°C until subsequent assays were performed for arachidonic acid metabolites. To ascertain whether the gold compounds augmented the catabolism of leukotrienes, [^{3}H]leukotriene B_4 and [^{3}H]leukotriene C_4 were incubated at 37°C for designated time intervals (0 - 60 min) with the gold compounds or carrier solvent. At the end of each incubation period, a constant amount of antibody to either leukotriene B_4 or leukotriene C_4 was added, the mixture cooled and radioimmunoassays for these arachidonic acid metabolites performed. Radioimmunoassay kits and/or reagents were purchased specifically for assaying the eicosanoids leukotriene B_4, leukotriene C_4 and prostaglandin E_2. The leukotriene B_4 radioimmunoassay was performed according to the procedure of Salmon et al. (1982). The protocols obtained from each manufacturer were used for

the leukotriene C_4 and prostaglandin E_2 radioimmunoassays. In general, to duplicate polypropylene tubes containing nonradioactive antigen (metabolite present in the cell supernatant, 0.1 mL), or standards (0.1 mL), were added radioactive metabolite ([^{3}H]leukotriene B_4, [^{3}H]leukotriene C_4 or [^{3}H]prostaglandin E_2, 0.1 mL), and antibody. The tubes were mixed and left to incubate at 4°C for 24 h. At the end of the incubation period, cold, activated charcoal was added to the tubes to absorb the free antigen remaining in the mixture. After centrifugation at 15,000 x g for 2 min, the supernatant (0.5 mL), containing the antigen- antibody complex, was mixed with ACS II (Amersham, Oakville, Ontario) scintillation cocktail and the radioactivity measured in a Beckman LS scintillation counter. The results obtained for the standards were used to construct a dose-response curve which was used to evaluate the results of the samples. Standard curves for the evaluation of results were constructed for each experiment. The cross-reactivity between the eicosanoid antibodies and arachidonic acid were minimal (approximately 0.005%) as were the cross-reactivities between the eicosanoids. Control studies in which cells were incubated with arachidonic acid, the gold compounds, cytochalasin B or carrier solvent, at concentrations used in the experiments,

showed no effect on the standard curve of each radioimmunoassay.

F. CELLULAR PRODUCTION OF SUPEROXIDE ANIONS

The neutrophil plasma membrane enzyme complex, NADPH oxidase, can be activated by a host of stimuli to reduce O_2 to the superoxide anion (O_2^-). O_2^- production was monitored continuously by its ability to reduce ferricytochrome c to ferrocytochrome c which absorbs strongly at 550 nm:

$$O_2^- + \text{cytochrome } c^{3+} \longrightarrow O_2 + \text{cytochrome } c^{2+}$$

Reaction mixtures consisted of 10^6 cells/mL in HBSS containing 10% fetal calf serum and 0.1 mM ferricytochrome c. Samples were assayed in 1 cm polystyrene cuvettes in thermostatted cell holders adjusted to 37°C in a Shimadzu UV-240 Spectrophotometer. After a background absorbance reading was taken at 550 nm, with a slit width of 1 nm, against a distilled water blank, the cell stimulus was added in a minimal volume and the change in absorbance was monitored continuously with a chart recorder. Superoxide generation rates were determined by the division of the rate of the absorbance change (calculated as the slope of the linear portion of the tracing), by the extinction coefficient (21/cm/mM)

for the absorbance difference between reduced and oxidized cytochrome c. Lag periods were calculated by extrapolating the slope=0 portion of the tracing to the time axis. Total O_2^- was determined by dividing the overall absorbance change by the extinction coefficient.

A cell-free system for generating O_2^- was employed to control for possible effects of the test agents on the ferricytochrome c reduction process. In this system, O_2^- was produced from the oxidation of xanthine (50 μM) by xanthine oxidase (0.0125 Units/mL).

G. ISOLATION OF THE GRANULE RICH FRACTION OF THE NEUTROPHIL

The granule rich fraction (GRF) of the neutrophil, shown to contain membrane profiles, vesicles and cytoplasmic granules (Hohn and Lehrer, 1975), was isolated using the method of Suzuki and Lehrer, (1980). Neutrophils were diluted to 50 x 10^6/mL in a 10 mM Hepes solution, pH 7.4, containing 0.035% BSA. The cells were stimulated for 10 min at 37°C with 10 nM TPA and the reaction terminated by the addition of three volumes of an ice cold sucrose solution (0.34 M). After centri- fugation at 400 x g for 10 min at 4°C, the cells were resuspended in the cold sucrose solution to attain a concentration of 10^8 cells/mL. The cells were homogenized in melting ice for 5 min and

centrifuged (400 x g, 4°C, 10 min), to remove unbroken cells and coarse cell fragments. The supernatant was diluted ten-fold and subjected to high speed centrifugation (20,000 x g, 4°C, 20 min) in a Sorvall RC-5B Superspeed centrifuge to obtain the GRF. The pellet (GRF) was resuspended in enough 0.34 M sucrose to attain 10^8 neutrophil equivalents/mL and was either used immediately in a cytochrome c assay or was stored at -70°C for future use.

Spectrophotometric assays were performed at 20°C in 1.5 mL polystyrene cuvettes containing 0.15 mL GRF; 0.05 mL of 0.2 M sodium phosphate, pH 7.0; 0.1 mL 1 mM ferricytochrome c in HBSS and 0.05 mL of 2 mM NADPH in 5 mM sodium phosphate, pH 8.0 or 0.34 M sucrose (control). At 5 min time intervals (up to 30 min), 0.5 mL of distilled water was added to each cuvette, the contents mixed, and the absorbance at 550 nm read on a Shimadzu UV-240 Spectrophotometer. Control studies performed in the absence of the GRF showed a very small reduction of ferricytochrome c due to NADPH. This was corrected for in the actual experiments which involved the GRF.

H. INTRACELLULAR CALCIUM DETERMINATIONS

The Quin 2 fluorescence probe was utilized to monitor intracellular calcium changes that arise when the neutrophil is stimulated by various agents. Quin

2 is a fluoresecent tetracarboxylate anion which undergoes an approximate five-fold increase in fluorescence upon the binding of calcium which it chelates with a 1:1 stoichiometry (Tsien et al., 1982a, 1984). Quin 2 is a highly charged molecule and does not readily cross the plasma membrane. The esterified derivative of this molecule, Quin 2-acetoxymethyl ester (Quin 2/AM), being uncharged and lipid soluble, readily penetrates the plasma membrane and once inside the cell, is cleaved back to the lipid insoluble Quin 2 by cytosolic esterases.

Recently, a new fluorescent calcium indicator, Fura 2, was developed, which, like Quin 2, was designed to measure calcium levels in intact cells (Grynkiewicz et al., 1985; Tsien et al., 1985; Poenie et al., 1985). As is the case with Quin 2, the cells are incubated with the membrane permeable tetraacetoxymethyl ester derivative, Fura 2/AM, and the ester is enzymatically cleaved once it gains entrance into the cell leaving the membrane-impermeable Fura 2. In comparison to Quin 2, Fura 2 exhibits significant improvements in its fluorescent properties upon complexing with Ca^{2+}. After prolonged exposure of cells to Quin 2, cellular cytotoxicity can be demonstrated. Due to its increased fluorescence intensity, Fura 2 can be used at one-tenth the Quin 2 concentration with no evidence of cellular

damage. Fura 2 also exhibits an increased selectivity for calcium, versus other divalent cations, and a lower affinity for calcium compared to Quin 2. (Quin 2 becomes saturated at calcium concentrations above 1 to 2 μM which are attained in the cytosol of activated cells.)

Neutrophils were diluted with HBSS to a concentration of 10^7/mL and loaded with either 10 μM Quin 2/AM or 1 μM Fura 2/AM (in Me_2SO, 0.25%) for 30 min at 37°C. Excess fluorescent indicator was removed by two washes with HBSS and the cells were resuspended in the same buffer at a density of 10^6/mL. Stock suspensions of loaded cells were kept at 20°C and aliquots were prewarmed to 37°C prior to assay. Fluorescence recordings were made on a Perkin Elmer model MKF-4 fluorescence spectrophotometer with the excitation wavelength set at 339 nm (5 nm slit width) and an emission wavelength set at 492 nm (15 nm slit width) in the case of Quin 2. With Fura 2, the excitation wavelength was set at 334 nm (5 nm slit width) and the emission wavelength set at 500 (20 nm slit width). Samples (2 mL of 10^6 cell/mL) were placed in 4.5 mL polystyrene cuvettes and stirred by a magnetic flea in a 37°C sample chamber. Concentrated test solutions were added to samples to minimize volume changes.

For the purposes of calculating intracellular

calcium concentration, the following equation was utilized:

$$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$$

(Tsien, et al., 1982a; Rink and Pozzan, 1985), where $[Ca^{2+}]$ is the calcium concentration, F is the fluorescence measured in the cell under test conditions and K_d is the approximate dissociation constant for calcium and Quin 2 (79-115 nM) or Fura 2 (135-224 nM). F_{max} was obtained by the addition of 0.1% Triton X-100 to the cells. F_{min} was obtained by treating cells with 2 mM EGTA in 50 mM Tris, pH 8.3, for extended periods of time until a minimal fluorescence reading was attained.

I. PROTEIN QUANTITATION

The Lowry method of protein estimation (Lowry et al., 1951) was adopted to analyze various samples prior to protein kinase C assay, binding experiments and electrophoretic analysis. To 0.1 mL of either sample or standard (BSA, 0.01 - 2 mg/mL) was added 0.1 mL of 2 N NaOH. Protein hydrolysis was carried out at 100°C for 10 min in a heating block. After cooling the hydrolysate to room temperature, 1 mL of freshly prepared complex-forming reagent (2% Na_2CO_3; 1%

$CuSO_4 \cdot 5H_2O$; 2% sodium potassium tartrate, 100:1:1 (v/v/v), respectively), was added. The solution was left at room temperature for 10 min at which time 0.1 mL of 1 N Folin-Ciocalteu reagent was added. After sufficient mixing, a 45 min incubation at room temperature was allowed. Absorbance readings of each duplicate sample and standard were made on a Shimadzu UV-240 Spectrophotometer at a wavelength of 750 nm. A standard curve of absorbance as a function of initial protein concentration was made and used to determine the unknown protein concentrations of the samples.

J. CELLULAR FRACTIONATION

For various studies it was necessary to separate the neutrophil cytosolic and particulate material using cellular disruption and ultracentrifugation. Neutrophils (2-10 x 10^7/mL) were resuspended in extraction buffer (25 mM Tris HCl, pH 7.4; 1 mM EDTA; 2 mM PMSF; 0.25 M sucrose; ± 0.1 mM leupeptin; ± 10 mM β-mercaptoethanol; ± 1 mM EGTA), following the last HBSS wash during neutrophil isolation. The cells were immediately placed on ice and disrupted by six, 10 s bursts (50-70 Watts) from a Braunsonic Model 1510 sonicator. In some experiments, the cells were treated with test reagents for various times prior to cellular disruption. After pelleting the nuclei and unbroken

cells (500 x g, 10 min, 4°C), the cell homogenates were placed in 10 mL polycarbonate ultracentrifuge tubes and subject to 100,000 x g centrifugal force for 1 h at 4°C in a Beckman L2-65B ultracentrifuge. The supernatant or cell cytosol was either utilized directly or was stored at -70°C. The pellet, containing the plasma membrane, granular membrane, endoplasmic reticulum, mitochondrial membrane and other vesicular membranes (particulate fraction), was solubilized in extraction buffer containing 0.1% Triton X-100 and either utilized immediately or stored at -70°C.

K. PARTIAL PURIFICATION OF PLATELET PROTEIN KINASE C

Protein kinase C (E.C. 2.7.1.37) was partially purified from human platelets in the laboratory of Dr. Michael Walsh (University of Calgary, Calgary, Alberta) by the following method. All steps were performed at 4°C. Human platelets (approximately 100 units), obtained from the Red Cross Blood Bank, were lysed by freezing and thawing in 40 mM potassium phosphate, pH 7.0; 10 mM EDTA; 0.2 mM PMSF; 1 mM dithiothreitol and centrifuged at 15,000 x g for 30 min. Solid ammonium sulfate was added to the supernatant slowly with stirring to achieve a final saturation level of 58% and allowed to stand for 20 min before centrifugation at 15,000 x g for 20 min. The supernatant was discarded

and the pellet was dissolved in a minimum volume of 20 mM Tris HCl, pH 7.5; 1 mM EGTA; 0.5 mM dithiothreitol and dialyzed overnight against two changes (10 mL each) of the same buffer. The dialyzed sample was loaded, at a flow rate of 50 mL/h, onto a column (2.5 x 50 cm), of DEAE-Sephacel previously equilibrated with dialysis buffer. The column was washed with dialysis buffer until the absorbance at 280 nm returned to baseline, indicating the absence of protein in the eluant. Bound proteins were eluted with a linear gradient generated from 600 mL each of dialysis buffer and dialysis buffer containing 0.35 M NaCl. Selected fractions were assayed for calcium- and phospholipid-dependent phosphorylation of Type III-S histone (described herein). Protein kinase C eluted at approximately 0.16 M NaCl, well separated from its major substrate, P47, which was pooled separately and further purified by hydroxyl-apatite column chromatography (Imaoka et al., 1983). Protein kinase C-containing fractions were combined, glycerol and soybean trypsin inhibitor were added (10% (v/v) and 0.2 mg/mL, respectively), and stored in 1 mL aliquots in plastic Eppendorf vials at -70°C. Enzymatic activity was stable for at least two years.

L. PROTEIN KINASE C ASSAY

The cytosolic and particulate fractions of the

neutrophil, as well as partially purified platelet protein kinase C, were assayed for protein kinase C activity. The method employed was a modification of the assay described by Kishimoto et al., (1983a). The assay mixture contained 20 mM Tris HCl, pH 7.4; 10 mM $MgCl_2$; 0.36 mg/mL Type III-S histone; 20 µM [γ -^{32}P]ATP (50-100 cpm/pmol); 0.01% Triton X-100; ± 1.2 mM phosphatidylserine; ± 15.4 µM diolein; ± 0.2 mM $CaCl_2$; ± AUR (10^{-8} - 10^{-4} M); ± GST (10^{-8} - 10^{-4} M) and 50 µL of sample fraction in a total volume of 250 µL. A mixture of phosphatidylserine and diolein were mixed by sonication in Me_2SO prior to their addition to the assay. Histone phosphorylation was initiated by the addition of [γ -^{32}P]ATP to the other components of the assay. Reactions were terminated at timed intervals (0.5 - 60 min) by the simultaneous additions of 0.8% BSA and 15% trichloroacetic acid. The precipitate was collected by filtration on 2.5 cm glass microfiber filters (Whatman GF/F) and radioactivity was measured with a Beckman LS 6800 scintillation counter.

The amount of cell fraction varied from 10-100 µg protein per assay. Enzymatic activity was measured as the incorporation of ^{32}P into histone from [γ -^{32}P]-ATP. Protein kinase C activity was usually expressed as pmol ^{32}P incorporated/mg protein/min.

M. CELLULAR INCORPORATION OF $^{32}PO_4$

In order to observe endogenous protein phosphorylation in the neutrophil, it was necessary to sustain the cells in a medium which was depleted of PO_4. In this starved state, the neutrophil would take up exogenous $^{32}PO_4$ into its PO_4 pool, and use it in subsequent phosphorylation reactions. Neutrophils were adjusted to 10^7/mL in a PO_4-free Hepes buffer (10 mM Hepes, pH 7.2; 0.14 M NaCl; 5 mM KCl; 5.56 mM glucose; 0.33 mM $CaCl_2$). $^{32}PO_4$ was added to the cell suspension as $H_3{}^{32}PO_4$ (0.5 mCi/mL), and the mixture was incubated in a shaking water bath for 1 h at 37°C. The cells were gently mixed every 15 min by pipetting through a 1 mL Eppendorf pipette. At the end of the incubation, the excess $^{32}PO_4$ was removed from the cells by three washes in PO_4-free Hepes buffer. The cells were finally resuspended in HBSS at a concentration of 10^7/mL. Treatment with test reagents was performed in HBSS buffer at 37°C for various times. Reactions were terminated by centrifugation in an Eppendorf microfuge (15,000 x g) for 2 min and resuspending the cells in 1 mL of 15% trichloroacetic acid. The proteins were allowed to precipitate for 30 min at 4°C after which time they were collected by centrifugation (15,000 x g) and washed extensively with ice-cold HBSS. Each sample of phosphoprotein

precipitate was resuspended in 200 µL SDS sample buffer (62.5 mM Tris HCl, pH 6.8; 2.3% (w/v) SDS; 10% (w/v) glycerol; 5% (v/v) ß-mercaptoethanol; 95°C), and heated for 10 min at 95°C to allow sufficient solubilization by SDS. The samples were either stored at -70°C or utilized immediately for gel electrophoresis.

In order to describe the percentage of radioactive label in a given protein compared to the total number of counts applied to the gel, as well as to determine the extent of $^{32}PO_4$ incorporation, TCA-precipitable counts were made for each solubilized sample. Five µL of each sample in SDS sample buffer were added to 1 mL of 15% TCA and allowed to precipitate for 30 min at 4°C. The precipitates were collected by centrifugation (15,000 x g) and resuspended in scintillation cocktail followed by measurements of radioactivity in a Beckman LS scintillation counter. The TCA precipitable counts method indicated that a 1 h incubation of cells with $^{32}PO_4$ was maximal and that any further incubation led to an increased proportion of dead cells. Also, radioactive concentrations of $^{32}PO_4$ less than 0.5 mCi/mL were insufficient to obtain successful incorporation. When the cells were not mixed during the 1 h incubation, incorporation of $^{32}PO_4$ was less than maximal (i.e. that obtained with mixing).

N. IMMUNOLOGICAL DETECTION OF PROTEIN KINASE C

An immunoblot analysis technique was employed to measure protein kinase C levels and the integrity of this enzyme in various samples of neutrophil homogenates, cytosolic or particulate fractions. Polyclonal antibodies against purified protein kinase C were prepared by Dr. Peggy Girard (Emory University, Atlanta, Georgia), according to the method of Girard et al., (1986). Neutrophils (5 x 10^7 in HBSS) were treated with test reagents for various times at 37°C. At the end of each incubation period, the cells were centrifuged for 10 min at 400 x g, and then homogenized by resuspension in hot SDS buffer (a solution containing 2% (w/v) SDS; 10 mM Tris HCl, pH 7.5; 10 mM EGTA; 95°C), at a concentration of 5 x 10^7/mL. The homogenates were heated for an additional 10 min at 95°C. Cytosolic and particulate neutrophil fractions were prepared for immunoblotting by solubilizing the particulate fraction (100,000 x g pellet) directly in hot SDS buffer and adding 2% SDS to the cytosolic fraction (100,000 x g supernatant). The cell homogenates, cytosolic or particulate fractions, were subjected to SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Zeta-Probe blotting membranes. Each membrane was incubated for 12 h at 50°C in a solution containing 3% (w/v) casein in Tris-buffered saline (20 mM Tris HCl, pH 7.5; 0.15 M

NaCl), to block nonspecific binding sites. This was followed by a 2-3 h incubation with the antisera (diluted 1:400 in Tris-buffered saline containing 1% casein). The membrane was then exposed to 10 mL of ^{125}I-protein A (containing 2 x 10^6 cpm), for 1 h followed by extensive washing with Tris-buffered saline. The air-dried membrane was autoradiographed to locate the immunoreactive proteins.

O. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to analyze mixtures of phosphoproteins as well as to prepare samples of cell homogenates, cytosolic and particulate fractions for subsequent analysis with anti-protein kinase C antisera.

Using this technique, the proteins are reacted with the anionic detergent, SDS, to form negatively charged complexes. The amount of SDS bound by a protein, and the resulting charge on the complex, is roughly proportional to its size. By binding SDS, the proteins are generally denatured and solubilized. The protein-SDS complex forms a rod of a length in proportion to the molecular weight of the protein. This results in proteins, of either acidic or basic isoelectric point, forming negatively charged complexes which can then be separated on the bases of charge

differences and sizes by electrophoresis through a sieve-like matrix of polyacrylamide gel. SDS-PAGE employs the principle of isotachophoresis, which involves concentration of the sample into very small zones. This leads to a better separation of the various species in the sample. Isotachophoresis is achieved by introducing a stacking gel, of different pH than the resolving gel, on top of the resolving gel. The sample is introduced to the system at the stacking gel. Ions move toward the electrodes when an electric field is applied, however, at the pH in the stacking gel, the protein-SDS complex has a mobility intermediate between the chloride ions (present throughout the system), and the glycinate ions (present in the running buffer). Due to their small size, the chloride ions have the greatest mobility. The larger ions follow and concentrate into narrow zones in the stacking gel, but do not effectively separate there. Upon reaching the resolving gel, the mobilities of the zones change in the pH prevailing there and the glycinate ion front overtakes the protein-SDS complex zone to leave it in a uniform buffered electric field thus allowing for protein separation according to size and charge.

The system of buffers utilized for SDS-PAGE was taken from that described by Laemmli (1970). The gel apparatus used was the Protean II Multi-Gel Casting

Chamber and Multi-Cell Electrophoresis Tank obtained from Bio-Rad (Richmond, California). Gels prepared in this manner were composed of 12% acrylamide, pH 8.8 in the resolving gel and 4.5% acrylamide, pH 6.8 in the stacking gel. Polymerization of acrylamide and bisacrylamide was initiated by the addition of TEMED and ammonium persulfate. (The persulfate activates the TEMED leaving it with an unpaired electron. The resulting radical then reacts with an acrylamide monomer to produce a new radical which can then react with another monomer, etc., to build up a polymer. The bisacrylamide was utilized to form crosslinks between the polymer chains.) An electric field was applied using a 20 milliampere constant current from a Buchler 3-1500 constant power supply.

Samples were added to the system in wells made in the stacking gel during polymerization. Samples were diluted in SDS sample buffer, (approximately 20 - 100 µg protein in 50 µL buffer), and heated for 10 min at 95°C prior to their addition. The ß-mercaptoethanol in the buffer reduced intermolecular disulfide bridges and destroyed quarternary structure thereby separating protein subunits. It also reduced intramolecular disulfide bonds to ensure maximal reaction with SDS. Glycerol was necessary in the buffer to increase the density of the sample. Bromophenol blue dye (0.1%) was

added to each sample prior to loading on the gel which acted as an indicator of the position of the front of electrophoresis in the gel. The dye also indicated when the sample was acidic by turning yellow instead of blue. Molecular weight standards (10,000 - 100,000), were treated in the same manner as the samples and were used to indicate the relative positions of each standard to the gel front. A standard curve (log molecular weight versus distance travelled) was constructed and the relative mobilities of each sample protein read off the curve to estimate their molecular weights.

Gels were stained using Coomassie blue R-250 (0.04% w/v) in a solution of 0.4% methanol and 10% glacial acetic acid for 2 h. Destaining of the gels was accomplished by several changes of destain solution (10% methanol; 15% glacial acetic acid). Destained gels were dried in a Bio-Rad Model 483 Slab Dryer for 2-3 h set at 80°C after which they were autoradiographed by placing the dried gels in X-ray cassettes with two intensifying screens. Kodak XAR5 film was placed on top of each gel and the film was exposed at -70°C for the required length of time.

In some experiments, unstained gels were blotted onto nitrocellulose membranes (0.45 μm pore size) for better resolution of the protein bands and to separate the free $^{32}PO_4$ background from the phosphoproteins.

P. ELECTROPHORETIC TRANSFER OF PROTEINS TO MEMBRANES

The method of transferring proteins to membranes from polyacrylamide gels was used to obtain a thin replica of the original gel where the proteins were no longer trapped within the gel matrix. In this method, the proteins become freely accessible to further analysis by being bound to the surface of a membrane.

Electrophoresis gels (SDS-PAGE) were pre-equilibrated in transfer buffer (25 mM Tris HCl, pH 8.3; 192 mM glycine), for 30 min at room temperature prior to the commencement of electrophoretic transfer. Pre-equilibration was necessary to facilitate the removal of contaminating electrophoresis buffer salts, which if present, may have increased the conductivity of the buffer. A Bio-Rad Trans Blot Cell was utilized for the purposes of the transfer. The equilibrated gel was placed in a sandwich assembly composed of the following sequence of materials: Scotch Brite gel pad / pre-equilibrated filter paper / equilibrated gel / pre-equilibrated membrane (Zeta-Probe or nitrocellulose) / pre-equilibrated filter paper / Scotch Brite gel pad, all held in place by the gel holder apparatus. It was necessary to carefully rub the transfer membrane from side to side to push out all the air bubbles and displace excess liquid from between the gel and the membrane. The membrane was rubbed until a nearly

adhesive contact was obtained with the gel. The sandwich was placed inside the Trans-Blot tank which had been filled with transfer buffer. A constant current of 200 milliamperes was applied for 4-6 h at 4°C. After the transfer, the membrane was washed in cold running water for 2 h after which time it was ready for subsequent analyses.

Q. [^{3}H]PHORBOL 12,13-DIBUTYRATE BINDING STUDIES

A [^{3}H]phorbol 12,13-dibutyrate binding assay was developed to study the binding of this phorbol ester to its receptor, protein kinase C. Binding of [^{3}H]phorbol 12,13-dibutyrate to the cytosolic fraction of the neutrophil (obtained from 5 x 10^7 cells/mL in extraction buffer), or partially-purified platelet protein kinase C, was measured using a polyethylene glycol, precipitation assay (Sharkey et al., 1984). The binding reaction mixture (0.25 mL) contained 50 mM Tris HCl, pH 7.4; 1 mM $CaCl_2$; 75 mM magnesium acetate; 62.5 µg/mL phosphatidylserine; 2 mg/mL human gammaglobulin; [^{3}H]phorbol 12,13-dibutyrate, and 100 µg sample protein. Eppendorf microfuge tubes were used for all binding assays. Test reagents were added to the assay mixture and replaced by carrier solvent in the control experiments. In some experiments, the cytosolic or platelet material was pretreated for various times at

37°C with the test reagents. Reactions were initiated by the addition of sample protein to the other premixed components of the assay. Binding of the agonist to protein kinase C was allowed to take place over varying incubation periods at 37°C, subsequent to the addition of sample protein. Reactions were terminated by the addition of ice-cold, 15% (w/v) polyethylene glycol 6000 in 50 mM Tris HCl, pH 7,4, and were placed at 4°C for 1 h to permit precipitation. The tubes were then centrifuged for 15 min at 15,000 x g in an Eppendorf Microfuge. After centrifugation, 100 µL of the supernatant was removed from each tube for determination of the actual concentration of free [^{3}H]phorbol 12,13-dibutyrate. The remaining supernatant was removed by aspiration. The tip of the microfuge tube containing the pellet was cut off and transferred to a scintiallation vial. The radioactivity of both aliquots of supernatant and pellet were measured in scintillation fluid (toluene) by a Beckman LS 6800 scintillation counter after a 30 min mixing period.

To measure phorbol 12,13-dibutyrate saturation curves, the concentration of free [^{3}H]phorbol 12,13-dibutyrate was varied between 0.1 and 200 nM. Nonspecific binding was measured in control samples containing 30 µM cold phorbol 12,13-dibutyrate in addition to [^{3}H]phorbol 12,13-dibutyrate. Specific

binding was obtained by subtracting the nonspecific binding from the total binding obtained in the presence of [^{3}H]phorbol 12,13-dibutyrate without cold phorbol 12,13-dibutyrate. Displacement of the bound [^{3}H]-phorbol 12,13-dibutyrate was investigated using cold phorbol 12,13-dibutyrate (0.1 - 100 μM) and various concentrations of test reagents. K_i values obtained from the measured IC_{50}s (50% inhibitory concentrations), were calculated by using the relationship:

$$K_i = IC_{50}/(1 + L/K_d)$$

in which L is concentration of free [^{3}H]phorbol 12,13-di- butyrate and K_d is dissociation constant for phorbol 12,13-dibutyrate from the receptor. K_d values were evaluated using a Scatchard anaylsis of data obtained using the LIGAND PC program developed by Munson and Rodbard, (1980).

R. STATISTICAL ANALYSES OF DATA

For certain experiments, the data points have been expressed as the mean ± standard error of the mean (SE). To assess the significance of differences between means, the Student's paired "t" test was employed.

CHAPTER THREE

RESULTS

A. CELLULAR VIABILITY IN THE PRESENCE OF VARIOUS AGENTS.

Both the LDH assay and the trypan blue dye exclusion criteria for cellular viability were employed to monitor neutrophil status following treatment by various agents. After treatment with each of the agents; fMet-Leu-Phe (1 μM), TPA (0.1 μM), F^- (NaF, 18 mM), and cytochalasin B (5 μg/mL), the cells remained viable up to 90 min. After this time, although cellular shape had been altered in some of the treated cell populations, both the treated and control cell populations began to die off.

The gold compounds AUR and GST, as well as D-penicillamine, did not induce an increased rate of cellular demise compared to the control population. Whereas the control cells lasted approximately 90 - 120 min, the gold drug-treated populations remained viable up to 3 h. In fact, microscopic inspection revealed that the shape of the gold drug-treated population of cells remained intact compared to the control population of cells whose membranes appeared ruffled and damaged after this period of time.

B. THE EFFECT OF GOLD COMPOUNDS ON NEUTROPHIL DEGRANULATION.

Cellular release of either lysozyme or β-glucuroni-

dase was accomplished by co-stimulation of the cells with fMet-Leu-Phe (1 μM) and cytochalasin B (5 μg/mL). Cytochalasin B belongs to a family of low molecular weight fungal alkaloids known to exert a variety of effects on mammalian cells (Carter, 1967; Wessels et al., 1971), including potentiation of neutrophil secretion (Zurier et al., 1973; Naccache et al., 1977; Bently and Reed, 1981). Neutrophils stimulated with fMet-Leu-Phe, released approximately 40% of their lysosomal constituents compared to that released by fMet-Leu-Phe in the presence of cytochalasin B. In the presence of carrier solvent (0.1% ethanol), virtually no cellular release of lysozyme was detected (Table IV). However, a 30 min pretreatment of the cells with either AUR or GST caused a moderate release of lysozyme which was independent of drug dose. That this drug-induced release of lysosomal contents occurred with the specific granules exclusively, is substantiated by the lack of release of ß-glucuronidase under similar conditions when compared to control cells (Table IV).

The following study was undertaken to evaluate the effects of the gold compounds on the stimulated release (fMet-Leu-Phe + cytochalasin B) of both granular enzymes. Figures 7 and 8 illustrate the results of these experiments. All cells were incubated for 30 min at 37°C in the presence of gold compounds or carrier

Table IV: Neutrophil degranulation in the presence of gold compounds.

	Enzyme Release (%control[a] ± SE[b])	
	Lysozyme	ß-glucuronidase
negative control[c]	2.49 ± 0.99	15.22 ± 5.27
AUR 1.0 µM	36.82 ± 8.95	12.16 ± 3.35
AUR 2.5 µM	27.36 ± 11.94	ND
AUR 5.0 µM	31.34 ± 10.62	12.49 ± 3.83
AUR 10.0 µM	33.33 ± 0.05	12.97 ± 3.84
AUR 20.0 µM	22.89 ± 1.49	13.02 ± 2.87
GST 1.0 µM	33.80 ± 0.12	13.45 ± 4.31
GST 10.0 µM	29.58 ± 1.25	13.40 ± 4.79
GST 100.0 µM	33.80 ± 1.01	9.72 ± 1.44

[a]cells stimulated with 5 µg/mL cytochalasin B + 1 µM fMet-Leu-Phe for a total of 15 min were used as a positive control

[b]values represent the mean ± standard error (SE) of 5 determinations

[c]cells treated for 30 min at 37°C with 0.1% ethanol

ND = not determined

Figure 7: Effect of gold compounds on lysozyme release from stimulated neutrophils.

—Cells were pretreated with either AUR (1-20 µM), GST (1-100 µM), or carrier solvent (control), for 30 min at 37°C and then stimulated with fMet-Leu-Phe (1 µM) and cytochalasin B (5 µg/mL). Lysozyme release was measured as described in MATERIALS AND METHODS. Results are the mean ± standard error of five determinations.

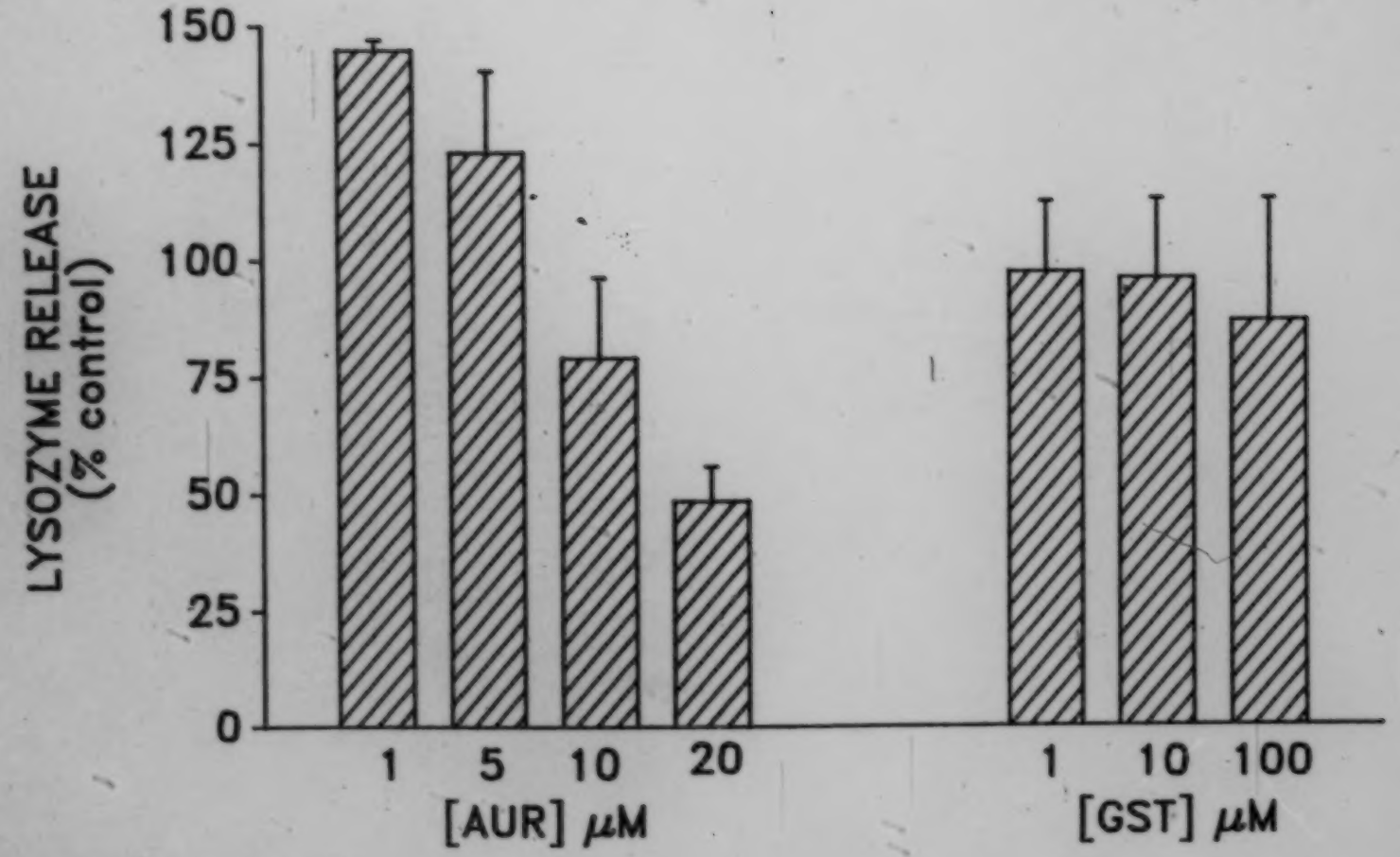
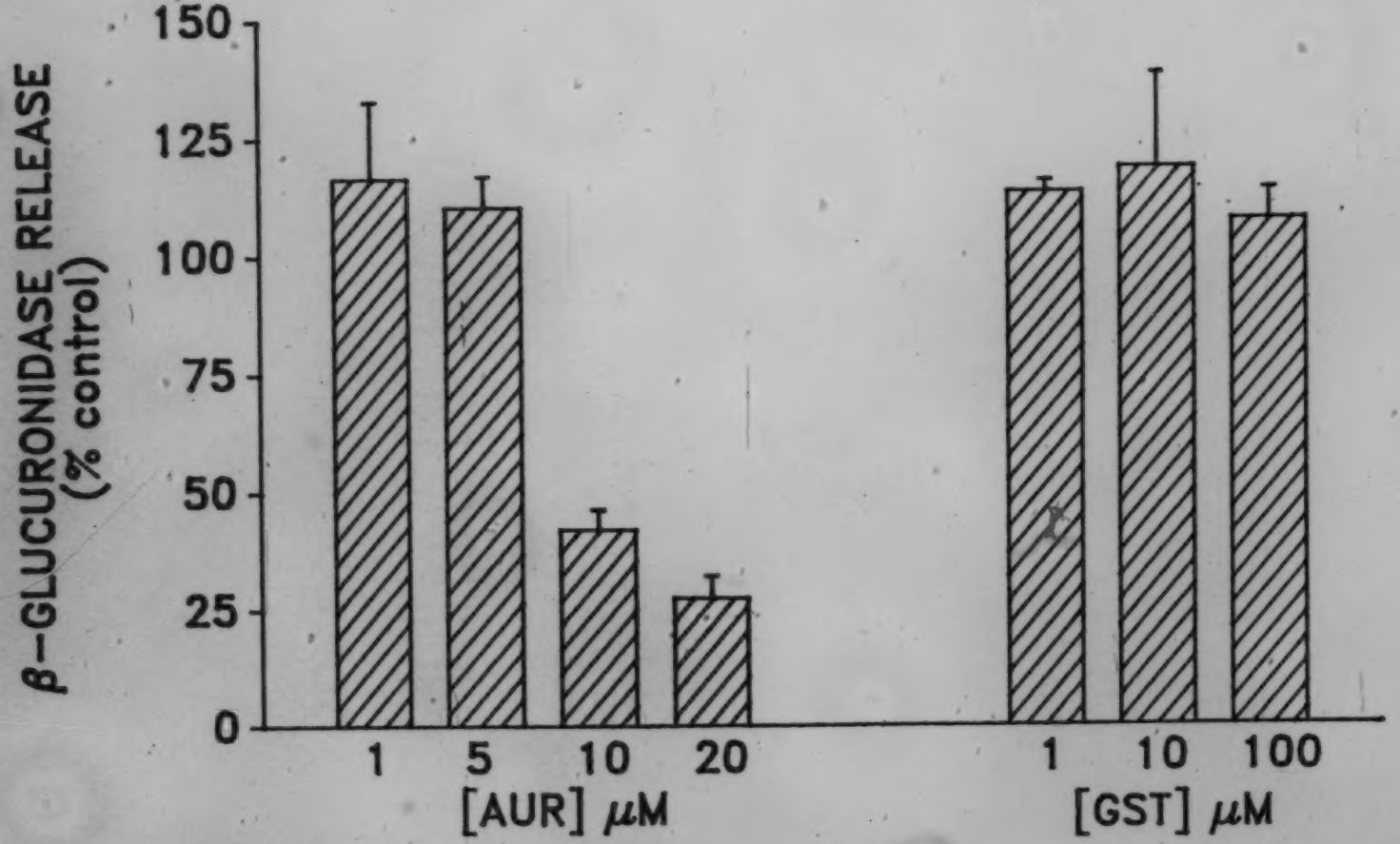

LYSOZYME RELEASE
(% control)
150
125
100
75
50
25
0
1
5
10
20
[AUR] μM
1
10
100
[GST] μM

Figure 8: Effect of gold compounds on β-glucuronidase release from stimulated neutrophils.

Cells were pretreated with either AUR (1-20 μM), GST (1-100 μM), or carrier solvent (control), for 30 min at 37°C and then stimulated with fMet-Leu-Phe (1 μM) and cytochalasin B (5 μg/mL). β-glucuronidase release was measured as described in MATERIALS AND METHODS. Results are the mean ± standard error of five determinations.


β-GLUCURONIDASE RELEASE
(% control)
150
125
100
75
50
25
0
1
5
10
20
[AUR] μM
1
10
100
[GST] μM

solvent, then stimulated and the extracellular released enzymes assayed. AUR suppressed the stimulated release of both lysozyme and ß-glucuronidase. This response was dependent on the dose of the drug employed. At low doses of AUR, enhanced release of both granular enzymes was observed (1-5 µM), but became inhibitory at higher doses. GST showed no appreciable effect on the release of either lysosomal enzyme.

C. DE NOVO EICOSANOID SYNTHESIS IN THE PRESENCE OF GOLD COMPOUNDS.

Eicosanoids (arachidonic acid metabolites), are biologically active oxidation products of arachidonic acid. When arachidonic acid is released from its esterified stores in the cell plasma membrane, two main enzymatic pathways may utilize it to produce potent mediators of inflammation: The cyclooxygenase cascade catalyzes the production of prostanoids, and the lipoxygenase enzymes catalyze the synthesis of leukotrienes, hydroxyeicosatetraenoic acids and lipoxins (Needleman et al., 1986). Since the eicosanoids can be produced by the neutrophil at sites of inflammatory injury and have been shown to play an important role as mediators of acute inflammation, the proposed role of gold compounds in suppressing the production of these bioactive lipids by the neutrophil seems a novel

mechanism by which these compounds could exert their antiinflammatory effects. To test this hypothesis, an assay system was developed to maximally stimulate the neutrophil to produce arachidonic acid metabolites.

Leukotrienes and prostaglandins were quantitated by radioimmunoassay according to MATERIALS AND METHODS. Control studies showed that when leukotriene standards were incubated with gold compounds in the absence of cells, neither AUR nor GST exerted any effect on the amount of leukotriene metabolite bound to antibody compared to that bound in the absence of drug. As well, the gold compounds did not directly destabilize the metabolites. Time course studies indicated that the amount of leukotriene metabolite bound to antibody remained constant throughout the assay (up to 60 min).

When neutrophils were stimulated with fMet-Leu-Phe (1 μM) in the presence of cytochalasin B (5 μg/mL), an enhanced synthesis of eicosanoids was detected compared to cells stimulated by the chemotactic peptide alone (Table V). Cells were not activated to produce arachidonic acid metabolites when incubated solely with cytochalasin B. An incubation period of 10 min at 37°C, in the presence of cytochalasin B, followed by a further 5 min in the presence of fMet-Leu-Phe, was found to be sufficient for maximal cell stimulation. However, no detectable changes in metabolite synthesis or degrada-

Table V: The production of leukotriene C_4 (LTC_4) and prostaglandin E_2 (PGE_2) by neutrophils under varying conditions.

	Eicosanoid Production (ng/0.1 mL ± SE[a])	
	LTC_4	PGE_2
no stimulus[b]	0.032 ± 0.002	0.023 ± 0.013
cytochalasin B[c]	<0.025	0.028 ± 0.012
fMet-Leu-Phe[d]	0.026 ± 0.009	0.040 ± 0.020
cytochalasin B + fMet-Leu-Phe[e]	0.406 ± 0.082	0.700 ± 0.184

[a]values represent the mean ± standard error (SE) of 6 determinations

[b]cells (10^7/mL) were incubated with carrier solvent (0.1% ethanol) for 15 min at 37°C

[c]stimulation with cytochalasin B (5 µg/mL) for 10 min

[d]stimulation with fMet-Leu-Phe (1 µM) for 5 min

[e]co-stimulation with cytochalasin B and fMet-Leu-Phe (5 µg/mL, 10 min and 1 µM, 5 min, respectively)

tion were observed at incubation times longer than this. The amounts of leukotriene B_4, leukotriene C_4 and prostaglandin E_2 produced by co-stimulation with fMet-Leu-Phe and cytochalasin B, and detected by radioimmunoassay, were comparable to those detected using reverse-phase high pressure liquid chromatographic techniques under similar conditions (Clancy et al., 1983; Serhan et al., 1984).

The gold compound AUR failed to initiate the de novo synthesis of arachidonic acid metabolites from unstimulated neutrophils. However, the stimulated synthesis of lipoxygenase products was dramatically suppressed. Both leukotriene B_4 and C_4 production by stimulated cells was inhibited at concentrations of AUR greater than 2.5 μM (Figures 9 and 10), reaching maximal inhibition at approximately 20 μM. However, a biphasic effect by this drug was observed in that, at lower concentrations, AUR enhanced the production of these metabolites, although statistical significance was not attained for the number of studies made.

Similar studies carried out with GST neither enhanced nor suppressed the production of leukotrienes from the stimulated cells at the concentrations tested. As well, this drug did not initiate the production of eicosanoids from unstimulated neutrophils.

The mean basal levels of leukotriene B_4 and

Figure 9: Effect of auranofin on the mean levels of leukotriene B_4 produced by activated neutrophils.

Cells were pretreated with AUR (2.5-20 μM) for 30 min at 37°C prior to stimulation with fMet-Leu-Phe (1 μM) and cytochalasin B (5 μg/mL). Leukotriene B_4 production was measured by radioimmunoassay according to MATERIALS AND METHODS. Results are the mean ± standard error of six determinations.

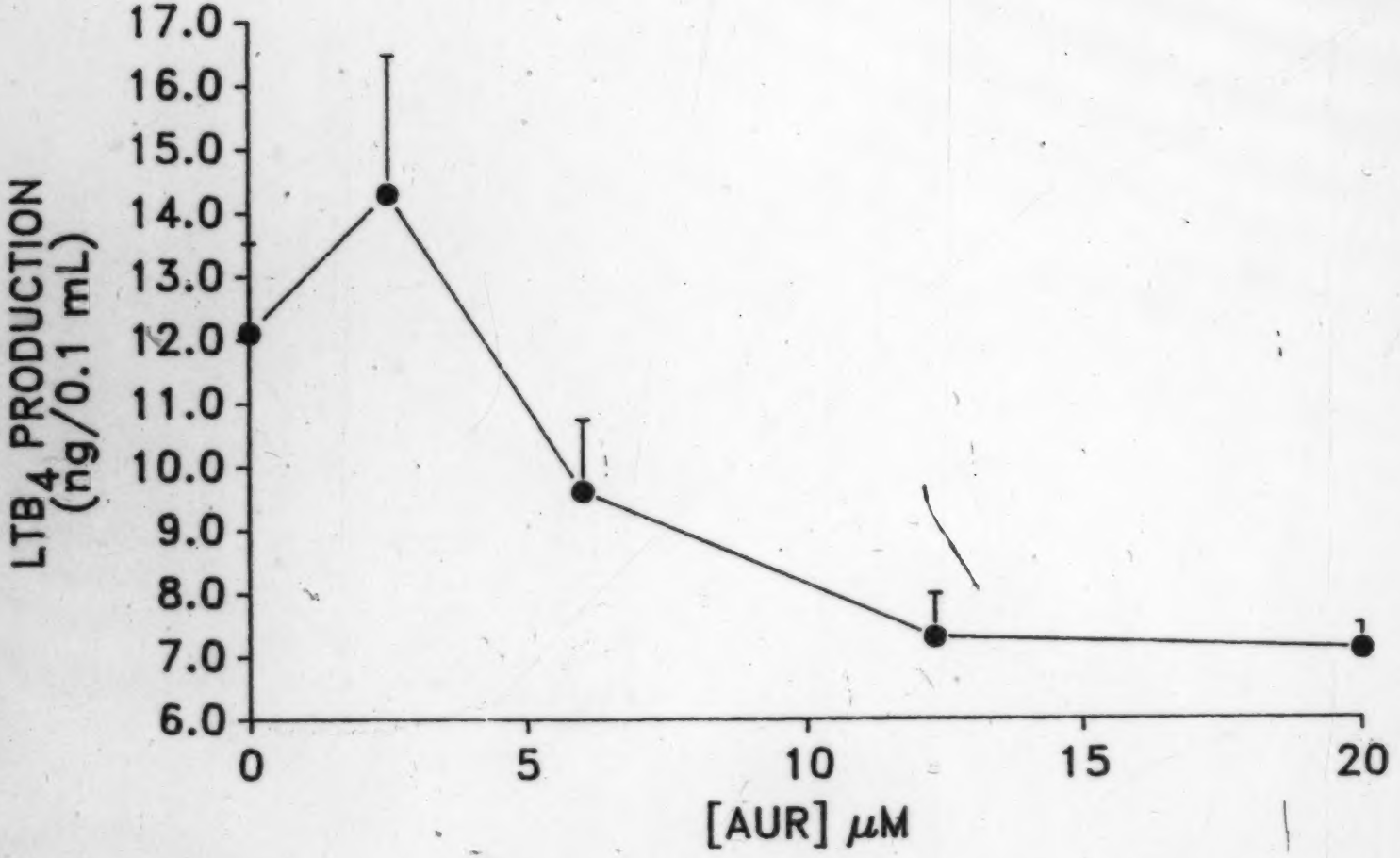
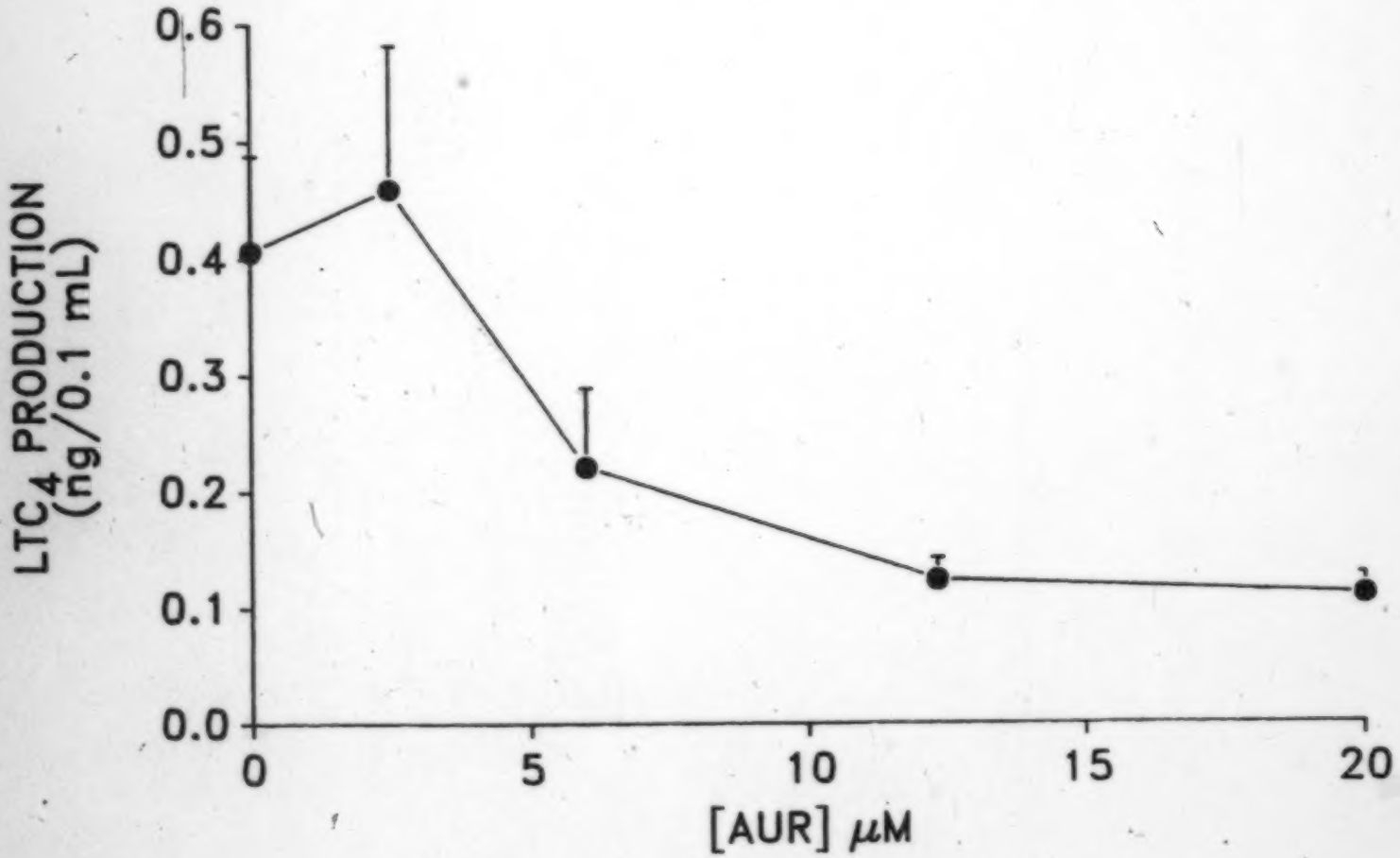

LTB4 PRODUCTION (ng/0.1 mL)
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
0
5
10
15
20
[AUR] μM

Figure 10: Effect of auranofin on the mean levels of leukotriene C_4 produced by activated neutrophils.

Cells were pretreated with AUR (2.5-20 μM) for 30 min at 37°C prior to stimulation with fMet-Leu-Phe (1 μM) and cytochalasin B (5 μg/mL). Leukotriene C_4 production was measured by radioimmunoassay according to MATERIALS AND METHODS. Results are the mean ± standard error of six determinations.


LTC4 PRODUCTION
(ng/0.1 mL)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
[AUR] μM

leukotriene C_4 present in the supernatant of control cells were <6.100 ng/0.1 mL and <0.032 ng/0.1 mL, respectively. These levels are representative of the lower sensitivity limits of the radioimmunoassays, that is, virtually no metabolites were formed by the resting cells.

Neither gold compound affected the de novo production of prostaglandin E_2, a cyclooxygenase product, from stimulated cells. The mean basal level of this metabolite was <24 pg/0.1 mL. AUR, at all concentrations tested, failed to alter the stimulated production of prostaglandin E_2 (Figure 11). Similar results were obtained with GST.

D. THE RESPIRATORY BURST INDUCED BY SOLUBLE STIMULI

The neutrophil respiratory burst, stimulated with fMet-Leu-Phe, TPA or F^-, was investigated and quantified with respect to lag period, the rate of superoxide anion production and the total yield of superoxide anion produced. Concentrations of stimuli providing optimal respiratory burst activity determined from previous studies (Wong and Chew, 1985; Strnad et al., 1986), were utilized in this study. Results are presented in Table VI and Figure 12.

A feature common to all of the stimuli employed, is the apparent time lag between the initial addition of

Figure 11: Effect of auranofin on the mean levels of prostaglandin E_2 produced by activated neutrophils.

Cells were pretreated with AUR (2.5-20 μM) for 30 min at 37°C prior to stimulation with fMet-Leu-Phe (1 μM) and cytochalasin B (5 μg/mL). Prostaglandin E_2 production was measured by radioimmunoassay according to MATERIALS AND METHODS. Results are the mean ± standard error of five determinations.

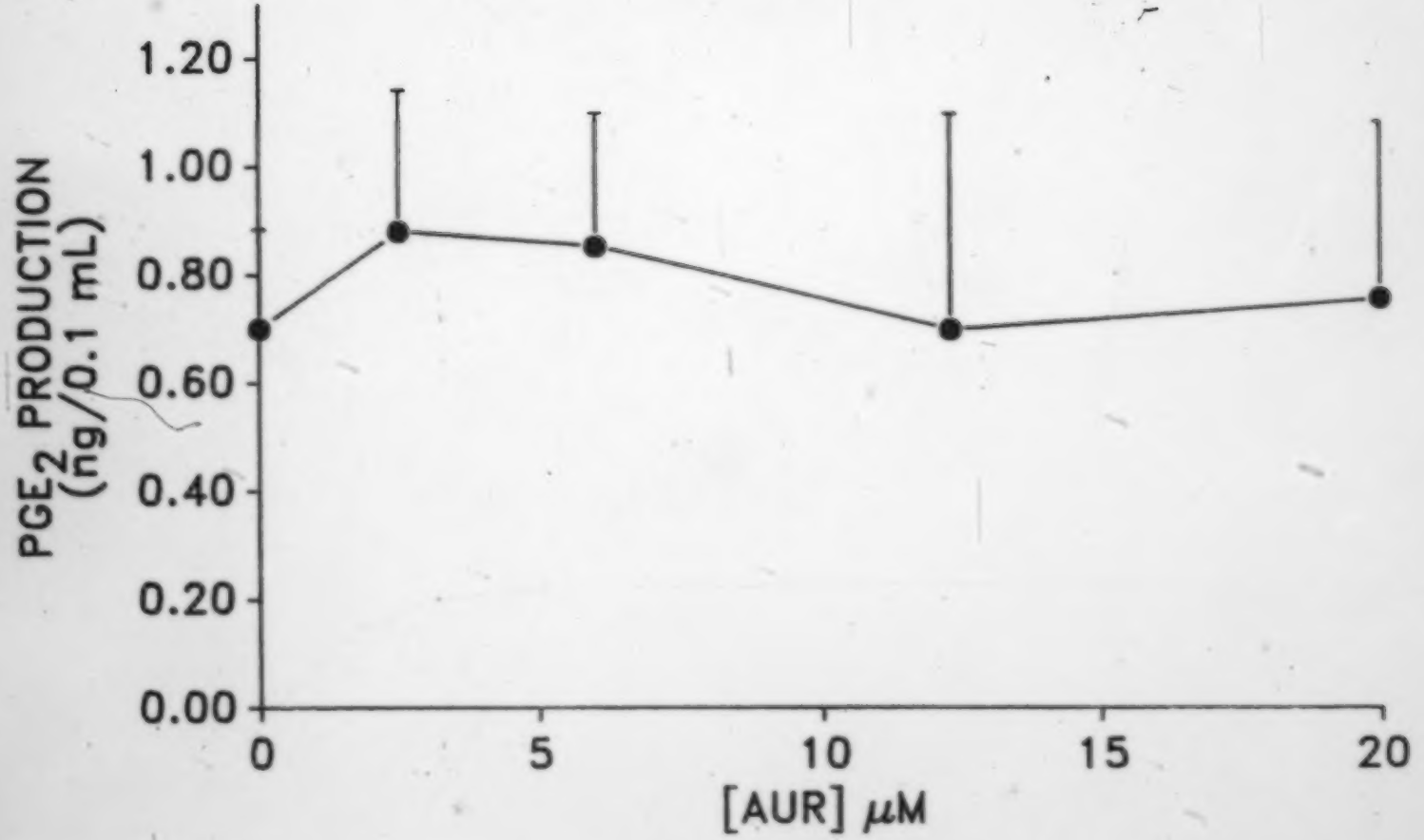

PGE2 PRODUCTION (ng/0.1 mL)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
5
10
15
20
[AUR] μM

Table VI: Characteristics of the respiratory burst activated by soluble stimuli.

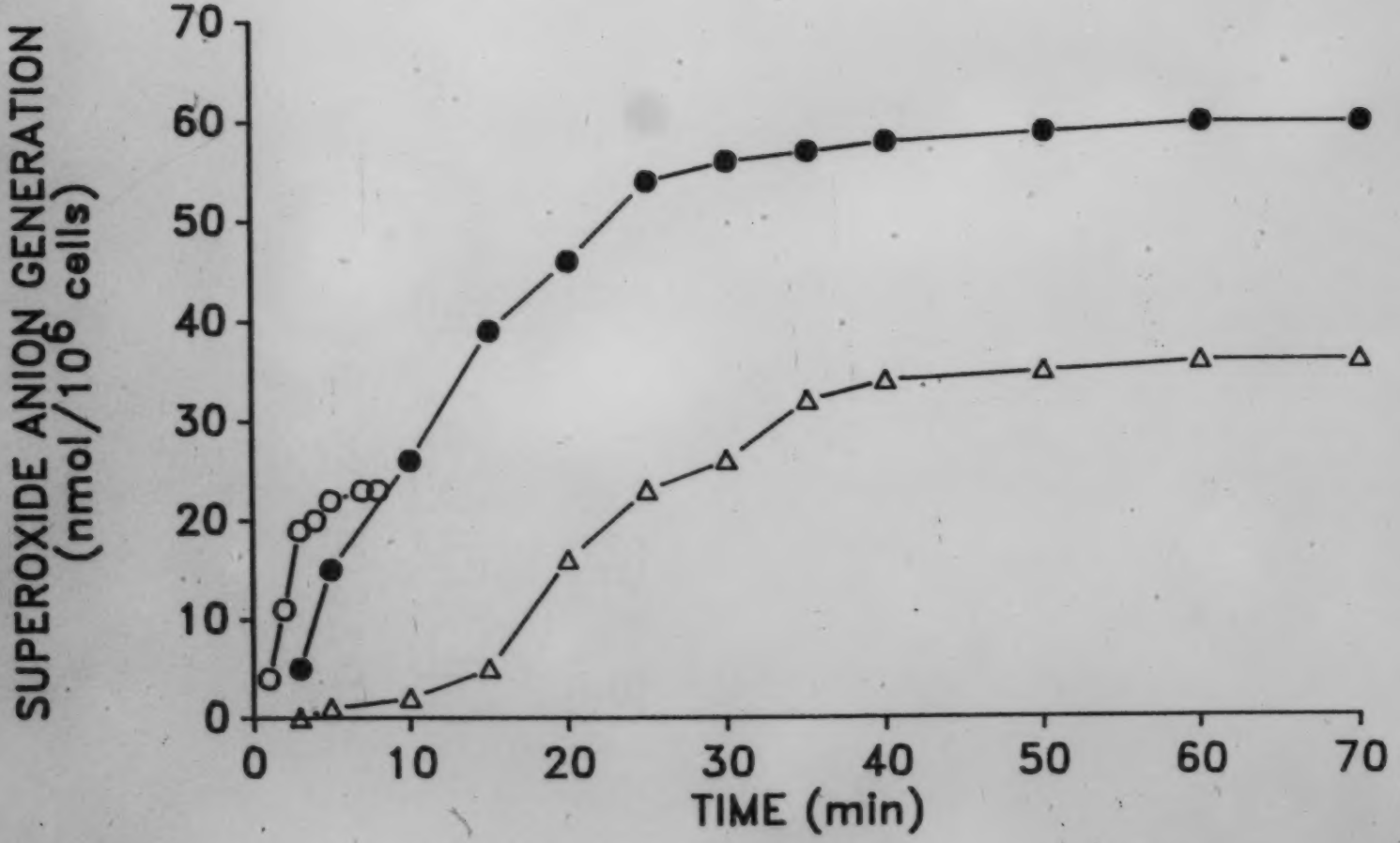
	n	lag	rate[a]	yield[b]
fMet-Leu-Phe				
1 µM	7	10-15s	9.2 ± 2.2	23.3 ± 3.3
TPA 0.1 µM	10	1-3 min	4.9 ± 0.7	60.0 ± 10.8
NaF 18 mM	7	10-15 min	2.0 ± 0.5	36.0 ± 5.7

[a] nmol/min/10^6 cells

[b] nmol superoxide anion/10^6 cells

Figure 12: Superoxide anion generation by neutrophils stimulated with fMet-Leu-Phe, TPA or F^-.

Neutrophils (10^6/mL) were stimulated with fMet-Leu-Phe (1 µM, ○), TPA (0.1 µM, ●) and F^- (18 mM NaF, △) and the production of superoxide anion measured by the reduction of ferricytochrome c according to MATERIALS AND METHODS.


SUPEROXIDE ANION GENERATION
(nmol/10^6 cells)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
TIME (min)

agent to the cell suspension and the production of superoxide anions, measured as the reduction of ferricytochrome c. After a lag period of approximately 10-15 s, 1 μM fMet-Leu-Phe elicited superoxide anion generation at a rate of 9 nmol/min/10^6 cells. This response peaked within 10 min yielding approximately 23 nmol of superoxide anion/10^6 cells. The superoxide anion generation rate for 0.1 μM TPA was approximately 5 nmol/min/10^6 cells. Compared to fMet-Leu-Phe, TPA demonstrated a longer lag period which is representative of the time necessary for incorporation of this agent into the lipid bilayer. Following the initial lag period, ferricytochrome c reduction proceeded steadily and levelled off after approximately 60 min. However, this plateau could represent the level at which ferricytochrome became limiting and not the maximum yield of superoxide anion generated (Wong and Chew, 1985).

The F^--induced respiratory burst was characterized by a lag period of 10-15 min and a duration of approximately 60 min. The rate of production of superoxide anion was much lower than that of fMet-Leu-Phe and TPA, although it yielded an amount of superoxide anion intermediate between the two stimuli.

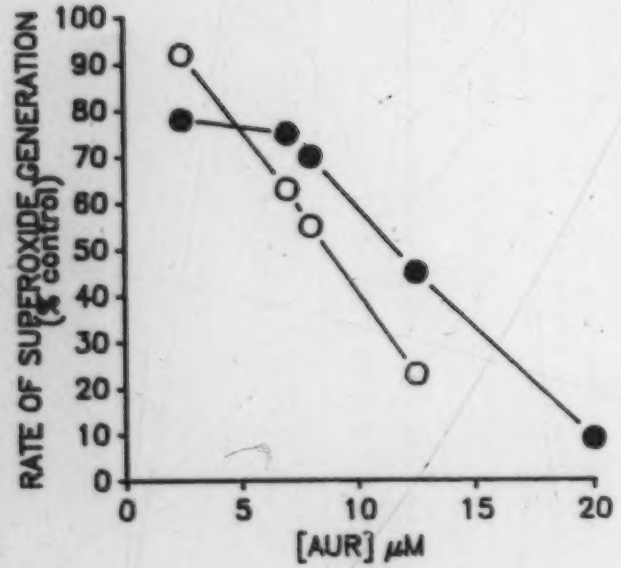
E. THE EFFECT OF GOLD COMPOUNDS ON THE NEUTROPHIL RESPIRATORY BURST.

Since the cellular stimuli, fMet-Leu-Phe, TPA and F^-, activate the neutrophil at different sites in the signal transduction pathway, the differential effect of gold compounds on responses induced by each of the three stimuli should provide clues as to the site of action of the former.

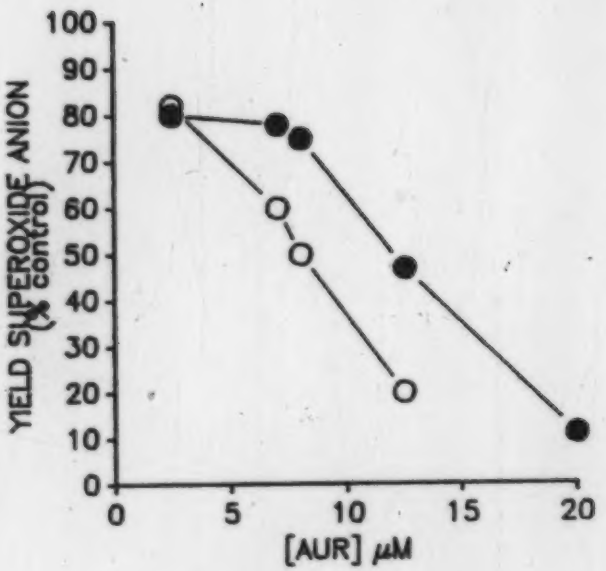
The gold compounds AUR and GST did not elicit a neutrophil respiratory burst when cells were treated with the compounds for up to 1 h. These experiments were performed in the absence of any agents known to stimulate the cells. Concentrations of the gold compounds ranged from 0.1 - 100 μM. In another set of experiments, neither gold compound influenced the generation of superoxide anions by quenching as demonstrated by control studies carried out in a cell-free system using xanthine/xanthine oxidase as a source of superoxide anions. However, the gold compound AUR did inhibit the rate and total yield of superoxide anion production induced by fMet-Leu-Phe and TPA (Figure 13). The mean rate of superoxide anion generation in fMet-Leu-Phe-treated cells was suppressed approximately 23% by 2.5 μM AUR. The rate was further suppressed as the concentrations of AUR increased to 20 μM. At similar concentrations of AUR, suppression of the mean

Figure 13: The effect of auranofin on the neutrophil respiratory burst induced by fMet-Leu-Phe and TPA.

The rate of superoxide anion generation, expressed as the percentage of control rate (in the presence of stimulus alone), is shown in panel A. The total yield of superoxide anion is shown in panel B, expressed as percentage of control yield. TPA (2 nM, O) and fMet-Leu-Phe (1 μM, ●) were added to the cells after a 30 min preincubation with AUR at 37°C. Each point represents the mean of 10 determinations with less than 5% standard error of the mean.


RATE OF SUPEROXIDE GENERATION
(% control)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
[AUR] μM

A


YIELD SUPEROXIDE ANION
(% control)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
[AUR] μM

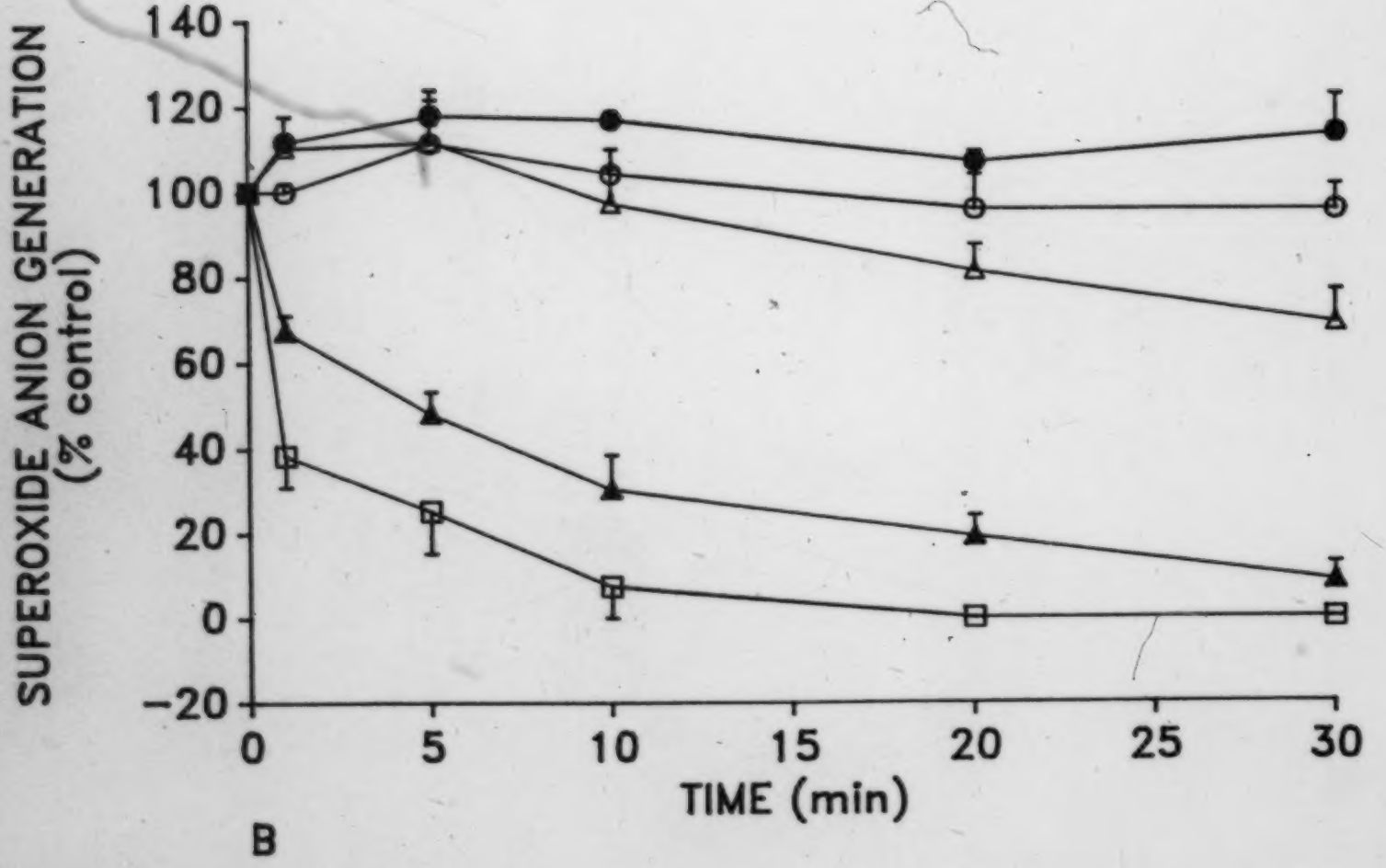
B

rate of superoxide anion generation was more pronounced when cells were activated by the phorbol ester, TPA. In both fMet-Leu-Phe- and TPA-treated cells, the percent inhibition by AUR of the mean total yield of superoxide anion displayed a similar pattern to that of the rate of generation. Again, TPA-treated cells displayed a greater degree of inhibition by AUR. In contrast to AUR, GST, at concentrations of 5 - 500 μM, failed to inhibit the rate or the total yield of superoxide anion from cells stimulated with fMet-Leu-Phe or TPA. In this study, the cells were treated with the gold compounds for a set incubation period of 30 min at 37°C. Expanding on these results, the effect of incubation time of various drug concentrations on the respiratory burst stimulated by fMet-Leu-Phe, TPA and F^- was investigated. The results indicated that AUR suppressed the respiratory burst rate in a time- and dose-dependent manner [Figure 14 (A, B, and C)]. AUR exerted a biphasic effect on superoxide anion generation induced in neutrophils by each of the stimuli. At lower AUR concentrations (0.1 - 1 μM), the rate of superoxide anion generation was slightly enhanced in fMet-Leu-Phe-stimulated cells [Figure 14 (A)], and was not inhibited with increasing time of exposure to the drug. A similar result was obtained in TPA- and F^--stimulated cells. However, AUR concentrations from 0.1 - 10 μM appeared to

Figure 14: Inhibition of the neutrophil respiratory burst by auranofin.

Neutrophils were preincubated in the presence of AUR for the indicated time intervals. Stimulatory agent was added and the rate of superoxide anion generation was calculated and compared to control rates. (A) stimulatory agent = fMet-Leu-Phe, 0.1 μM; (B) stimulatory agent = TPA, 0.1 μM; (C) stimulatory agent = NaF, 18 mM. AUR concentration (μM): (○) 0.1, (●) 1, (△) 10, (▲) 20, (□) 100. Each point represents the mean ± standard error of five determinations.

SUPEROXIDE ANION GENERATION
(% control)
180
160
140
120
100
80
60
40
20
0
−20
0
5
10
15
20
25
30
TIME (min)
A


SUPEROXIDE ANION GENERATION
(% control)
140
120
100
80
60
40
20
0
−20
0
5
10
15
20
25
30
TIME (min)

B

SUPEROXIDE ANION GENERATION
(% control)
180
160
140
120
100
80
60
40
20
0
−20
0
5
10
15
20
25
30
TIME (min)
C

enhance the TPA-mediated response [Figure 14 (B)], whereas, enhancement of the F^--induced response was demonstrated by the lower concentrations of AUR (0.1 - 1 μM), [Figure 14 (C)]. Higher concentrations of AUR inhibited the reaction in dose-dependent manner, with all stimuli employed. The extent of inhibition was also dependent on the exposure time of cells to AUR prior to the addition of stimulus.

On initial inspection, it appears that the F^--induced generation of superoxide anions was more sensitive to inhibition by AUR that the fMet-Leu-Phe- and TPA-mediated responses. However, with the concentration of stimuli used in this study, the lag period for fMet-Leu-Phe- or TPA-induced responses is far less that that for F^- (refer to Table VI). Therefore, the period of exposure of neutrophils to AUR is actually longer that is apparent in Figure 14(C).

Echoing previous findings in intact cells, GST was much less potent as an inhibitor of the respiratory burst compared to AUR. Weak inhibition by GST was detected at concentrations greater than 0.5 mM.

F. THE EFFECT OF GOLD COMPOUNDS ON SUPEROXIDE ANION GENERATION BY THE NEUTROPHIL GRANULE-RICH FRACTION.

The granule-rich fraction was isolated from TPA-activated neutrophils to assess the possibility of AUR

having a direct action on the NADPH oxidase enzyme complex.

In the absence of NADPH, the granule-rich fraction showed no detectable generation of superoxide anions as measured by the ferricytochrome c assay. This confirms the presence of NADPH oxidase activity in the granule-rich fraction. TPA preactivation of cells was also a prerequisite for superoxide anion generation. That is, the granule-rich fraction isolated from unstimulated neutrophils was unable to reduce ferricytochrome c above and beyond the reduction detected by NADPH alone. (Control studies performed in the absence of the granule-rich fraction, showed a very small reduction of ferricytochrome c due to NADPH. This was corrected for in the actual experiments involving the granule-rich fraction.)

The granule-rich fraction isolated from cells pretreated with either AUR (20 μM) or GST (0.5 mM), showed a similar rate of superoxide anion generation compared to that of the control (0.1% ethanol). In another study, the direct effect of the gold compounds on the granule-rich fraction isolated from untreated cells, was investigated. The results indicated that both gold compounds failed to affect the rate of generation of superoxide anion by the granule-rich fraction (Table VII). The difference in the means of

Table VII: Effects of gold compounds on the generation of superoxide anions from the granule-rich fraction of neutrophils.

	Rate of superoxide anion production[a]
control - no NADPH	0
gold compounds added to intact cells[b]	
no drug	3.00 ± 0.50
AUR (20 μM)	3.20 ± 0.50
GST (0.5 mM)	3.30 ± 0.40
gold compounds added to isolated granule-rich fraction[c]	
no drug	1.00 ± 0.16
AUR (20 μM)	0.97 ± 0.20
GST (0.5 mM)	0.90 ± 0.10

[a] nmol/min/10^6 cell equivalents. Intact cells were activated with TPA (0.1 μM, 10 min) and the granule-rich fraction isolated and assayed as described in MATERIALS AND METHODS. Results are the mean ± standard error of 10 determinations

[b] the granule-rich fraction was isolated from cells which had been pretreated with the gold compounds for 30 min at 37°C prior to activation with TPA

[c] the gold compounds were added directly to the granule-rich fraction obtained from untreated cells

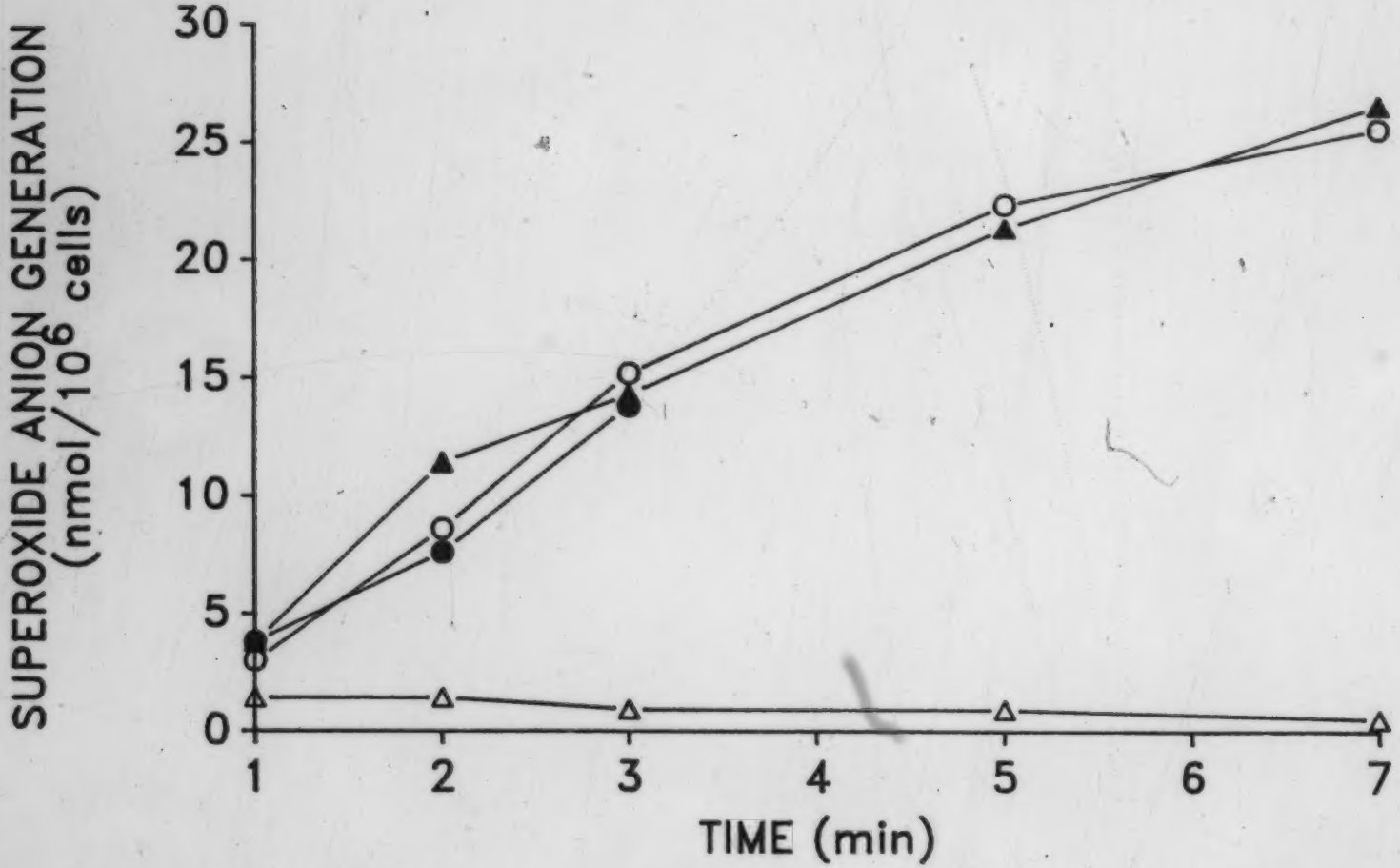
the rates of superoxide anion production between the granule-rich fractions of gold drug-treated and untreated cells, reflects individual variations existing among the two groups of cell populations tested. These values are much lower than one would expect from intact cells (refer to Table VI). Previous investigators (Light et al., 1981; Wong and Chew, 1985), have attributed this finding to the labile nature of the granule-rich fraction and to the loss of active material which occurs during its preparation.

G. REVERSAL OF AURANOFIN-MEDIATED INHIBITION OF THE RESPIRATORY BURST BY DITHIOTHREITOL.

In an attempt to demonstrate a dependence of AUR-mediated inhibition of the respiratory burst on intracellular free sulfhydryl groups, the thiol protective agent, dithiothreitol, was employed. Figure 15 illustrates the reversal of AUR's inhibitory action on the generation of superoxide anions elicited by fMet-Leu-Phe. A complete attenuation of the response was demonstrated in cells preincubated with 20 μM AUR for 5 min. Preincubation of cells with 20 μM AUR and 1 mM dithiothreitol resulted in a reversal of AUR-induced inhibition and a response equivalent to that elicited by fMet-Leu-Phe in control cells. Dithiothreitol alone did not affect superoxide anion production in stimulated

Figure 15: The effect of dithiothreitol on the inhibition of the respiratory burst mediated by auranofin.

Neutrophils were pretreated for 5 min at 37°C with 0.1% ethanol (○), 20 μM AUR (△), 1 mM dithiothreitol (●), or 20 μM AUR and 1 mM dithiothreitol (▲), prior to stimulation by 0.1 μM fMet-Leu-Phe. Superoxide anion production was measured its ability to reduce ferricytochrome c as described in MATERIALS AND METHODS.


SUPEROXIDE ANION GENERATION
(nmol/10^6 cells)
30
25
20
15
10
5
0
1
2
3
4
5
6
7
TIME (min)

cells. The results of this study are consistent with those of Roisman and coworkers (1983) wherein dithiothreitol reversed the inhibition mediated by AUR of the immune complex-induced neutrophil respiratory burst.

H. INTRACELLULAR CALCIUM MEASUREMENTS IN STIMULATED NEUTROPHILS AND THE EFFECT OF GOLD COMPOUNDS.

As reported in the previous section, the TPA-induced respiratory burst was suppressed in a time- and dose-dependent manner by AUR. Since TPA activates protein kinase C, it is suggested that the gold drug inhibits the generation of superoxide anion at this level. However, it is necessary to assess whether early signal transduction steps, such as activation of G-proteins and phosphoinositide turnover, are being compromised by AUR. Since the activation of these early steps ultimately leads to the mobilization of intracellular calcium, this present study was undertaken to test the effects of the gold compounds, especially AUR, on the levels of intracellular calcium in resting and stimulated cells. Measurements of intracelluar calcium concentration were made by use of the fluorescent calcium chelators, Quin 2/AM and Fura 2/AM.

Upon exposure to 1 μM fMet-Leu-Phe or 18 mM NaF, neutrophils respond with an increase in the levels of cytosolic free calcium. The fMet-Leu-Phe-induced

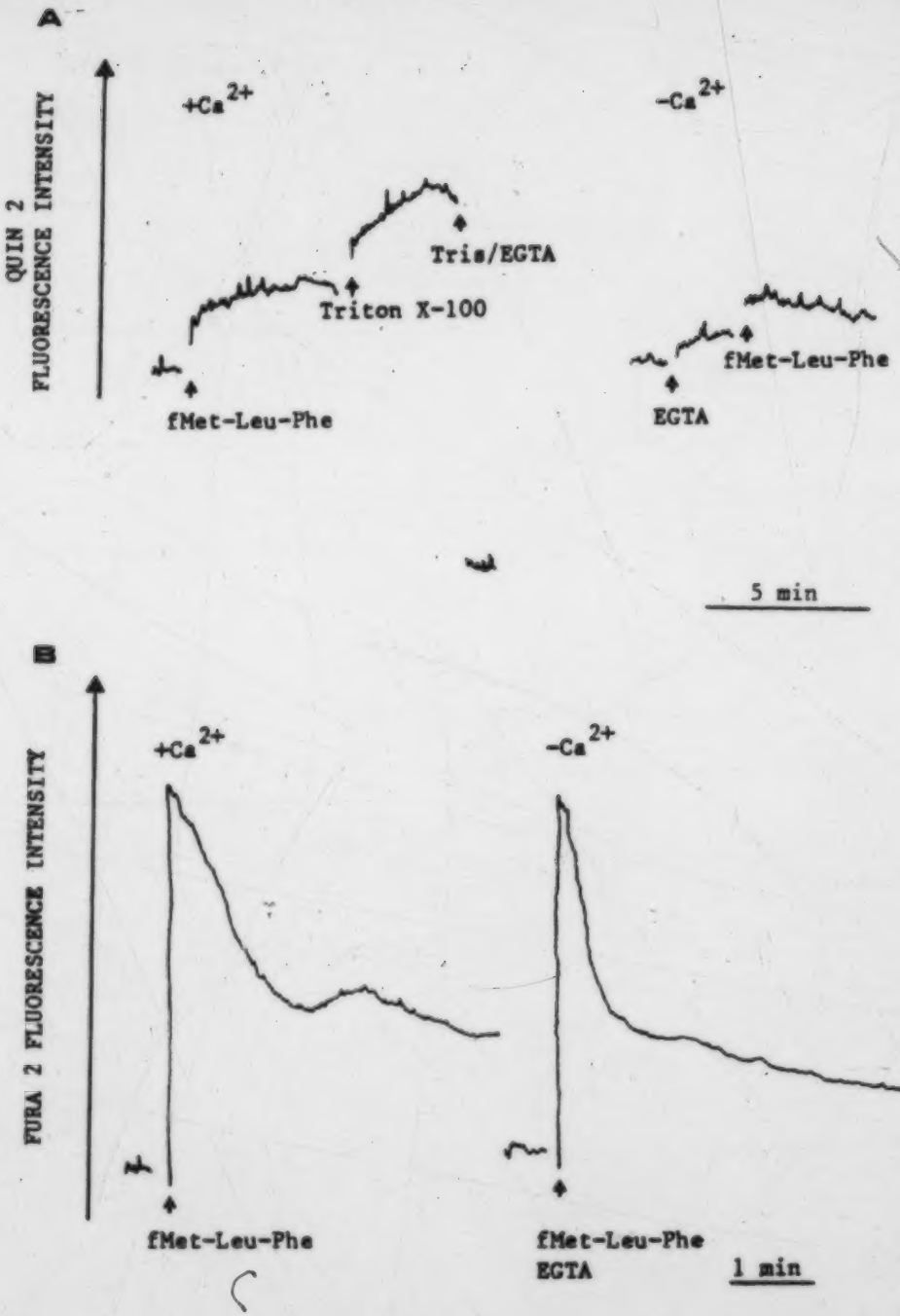
increase in cytosolic calcium concentration is characterized by a very rapid onset which plateaus and declines in the course of 7-10 min [Figure 16(A)]. Upon closer inspection, using the fluorescent calcium probe, Fura 2/AM [Figure 16(B)], this influx of calcium occurs in two phases. The first phase occurs within seconds after the addition of fMet-Leu-Phe and represents the mobilization of intracellular calcium stores as it is insensitive to the extracellular addition of the calcium chelator, EGTA. The second phase is latent (after 2 min), and is sensitive to the removal of extracellular calcium. Thus, this phase represents the increase in intracellular calcium concentration due to an influx from the extracellular fluid.

Higher levels of intracellular calcium concentrations were attained in fMet-Leu-Phe-stimulated cells by the Fura 2 method as compared to Quin 2, (approximately 900 nM and 400 nM, respectively). This is due to the high affinity of Quin 2 for calcium which tends to become saturated at increased levels of calcium which are attained in the stimulated cell. Fura 2 has a lower affinity for calcium compared to Quin 2 and this effect is therefore not observed.

In response to stimulation with 18 mM NaF, there was a very slow but immediate rise in intracellular calcium levels in the neutrophil. Maximum calcium levels,

Figure 16: Intracellular calcium measurements in fMet-Leu-Phe stimulated neutrophils.

Fluorescence recordings of (A) Quin 2/AM- and (B) Fura 2/AM-loaded neutrophils. Cells were loaded with the fluorescent calcium probes Quin 2/AM and Fura 2/AM according to MATERIALS AND METHODS. Cells were stimulated with fMet-Leu-Phe (1 µM) in the presence ($+Ca^{2+}$) and absence ($-Ca^{2+}$, 2 mM EGTA) of extracellular calcium. In (A), measurements of F_{max} (0.1% Triton X-100) and F_{min} (2 mM EGTA/50 mM Tris) were made to calculate the intracellular calcium concentration.


A
QUIN 2
FLUORESCENCE INTENSITY
+Ca2+
-Ca2+
Tris/EGTA
Triton X-100
fMet-Leu-Phe
fMet-Leu-Phe
EGTA
5 min
B
FURA 2 FLUORESCENCE INTENSITY
+Ca2+
-Ca2+
fMet-Leu-Phe
fMet-Leu-Phe
EGTA
1 min

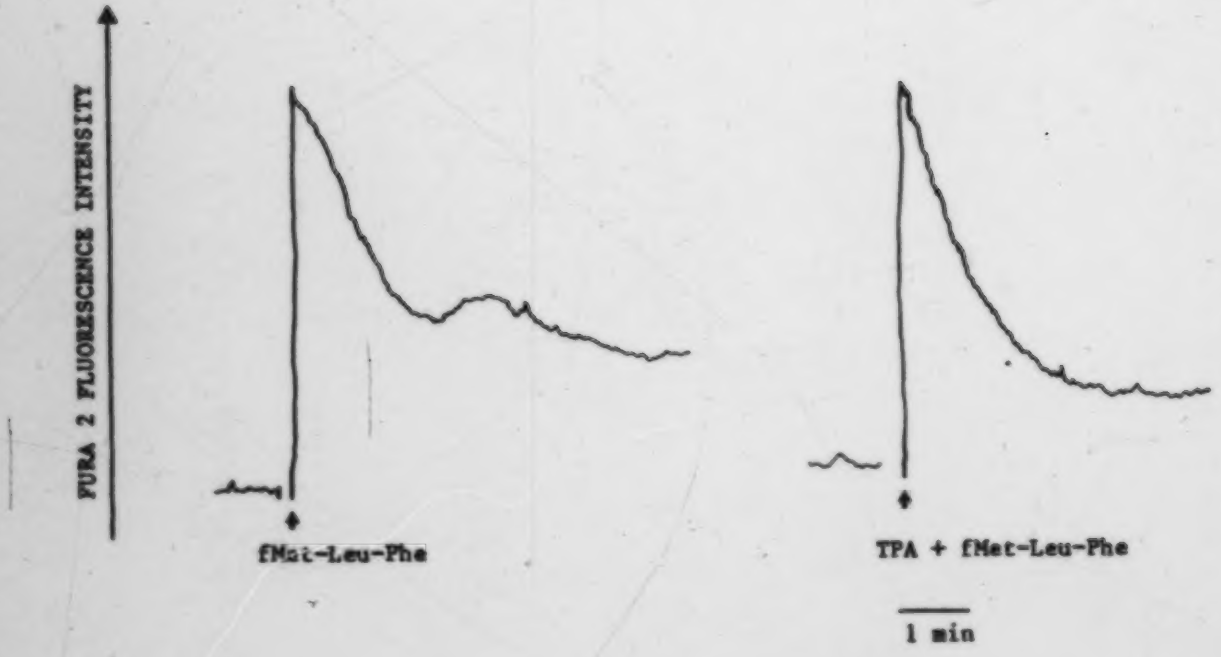
calculated using Fura 2, were at least 25% that obtained in cells stimulated with fMet-Leu-Phe.

The addition of TPA (0.1 μM) to Fura 2/AM-loaded cells prior to stimulation with fMet-Leu-Phe, had no effect on the fluorescence signal. However, upon the addition of fMet-Leu-Phe, a dramatic difference in the fluorescence patterns is obtained compared to that of fMet-Leu-Phe alone (Figure 17). In fact, the pattern observed is reminiscent of that obtained by the removal of extracellular calcium with EGTA. This is explained by the findings of various groups (Tsien et al., 1982b; Drummond, 1985; Sagi-Eisenberg et al., 1985), that, in various cell types, cytosolic calcium concentration is decreased by the addition of phorbol esters. It has been proposed that protein kinase C activates calcium-transport adenosine triphosphatase and thereby promotes the rapid extrusion of calcium from inside the cell.

Monitoring the transient increase in intracellular calcium concentration upon addition of fMet-Leu-Phe and F^- to cells in the presence of gold compounds provided interesting and valuable results. Preincubation of Fura 2/AM-loaded neutrophils with 20 μM AUR for 5 min, a concentration and time which inhibited the fMet-Leu-Phe- and F^--mediated respiratory burst, did not inhibit the increase in intracellular calcium concentration induced by either of these agents (Figure

Figure 17: The effect of phorbol ester on the fMet-Leu-Phe-induced changes in intracellular calcium levels.

Fluorescence recordings of Fura 2/AM-loaded neutrophils stimulated with fMet-Leu-Phe (1 μM) in the presence and absence of 0.1 μM TPA.


FURA 2 FLUORESCENCE INTENSITY
fMet-Leu-Phe
TPA + fMet-Leu-Phe
1 min

18). The second phase of calcium mobilization elicited by these agonists was, however, affected by AUR. On close inspection, AUR appeared to augment the calcium influx phase of the fluorescence signal. Thus, AUR promotes a greater influx of calcium into the cell, or alternatively, inhibits its efflux. The latter possibility could occur if AUR was inhibiting protein kinase C. It was previously demonstrated that TPA inhibited the second phase of the fMet-Leu-Phe response in a similar manner as EGTA. If AUR is inhibiting protein kinase C, then the active extrusion of calcium through the calcium-transport adenosine triphosphatase would also be inhibited resulting in the accumulation of calcium in the cytosol as demonstrated in Figure 18.

The addition of GST had no effect on either phase of the stimulated mobilization of intracellular calcium. Both increasing the concentration or the time of exposure of GST to cells did not alter the increases in intracellular calcium concentration induced by either F^- or fMet-Leu-Phe.

I. THE EFFECT OF GOLD COMPOUNDS ON INTRACELLULAR CALCIUM LEVELS IN RESTING NEUTROPHILS.

Upon addition to Quin 2/AM-loaded neutrophils, AUR induced a dose-dependent elevation in cytosolic calcium concentration (Figure 19). Unlike the response

Figure 18: Effect of AUR on calcium mobilization induced by fMet-Leu-Phe and F^-.

Neutrophils loaded with the fluorescent calcium indicator, Fura 2/AM, were exposed to 0.2 μM fMet-Leu-Phe, 20 μM AUR, or 18 mM NaF at points indicated by arrows. Fluorescence changes were monitored continuously and intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated according to the formula $[Ca^{2+}]_i = 224\ nM\ (F - F_{min} / F_{max} - F)$.

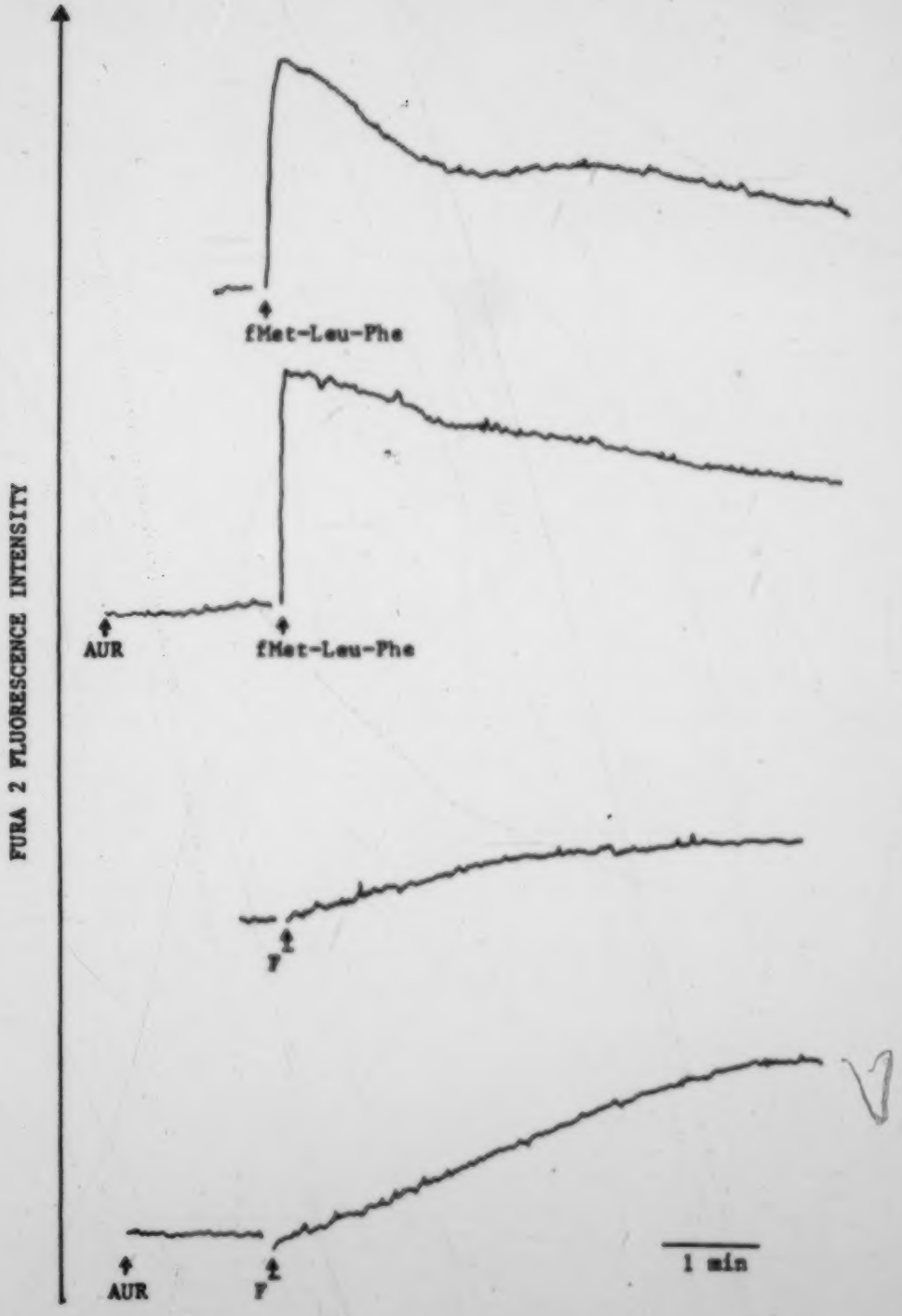
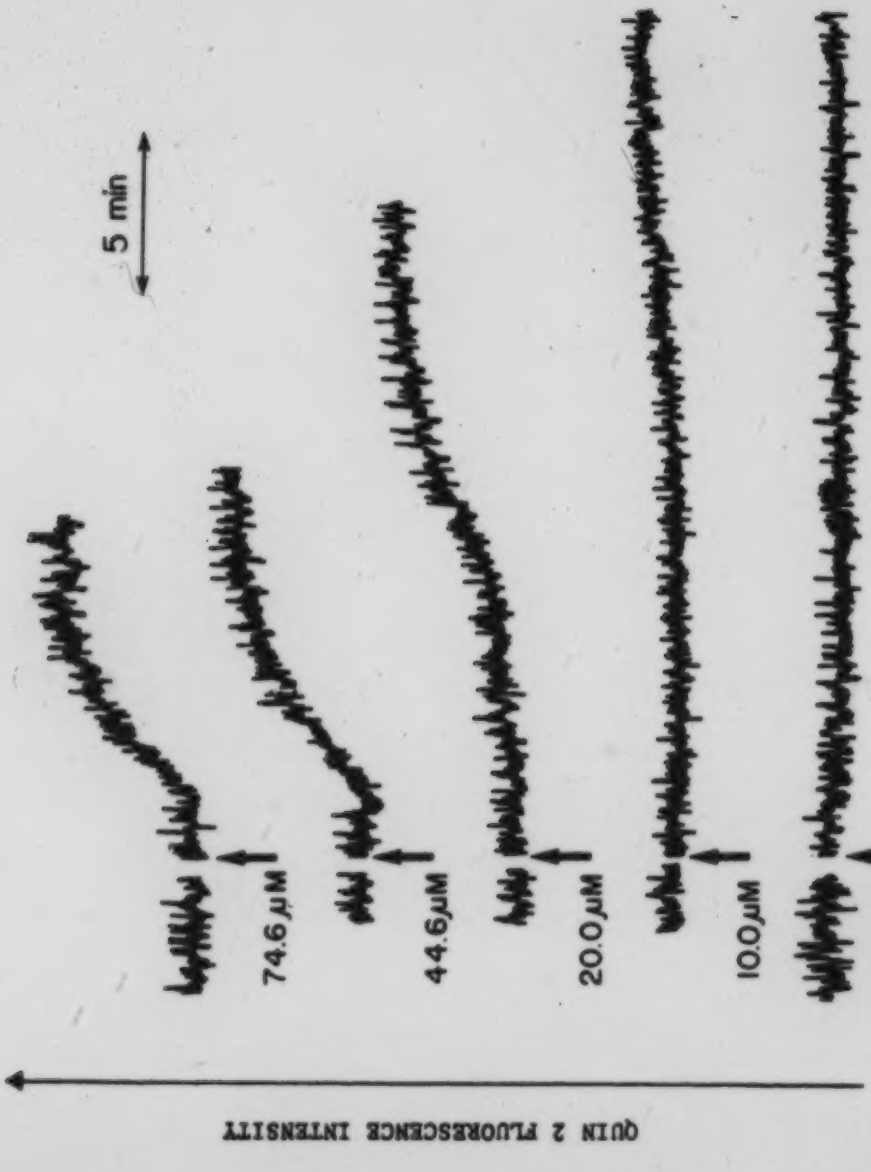

FURA 2 FLUORESCENCE INTENSITY
fMet-Leu-Phe
AUR
fMet-Leu-Phe
F
AUR
F
1 min

Figure 19: Auranofin induced dose-dependent changes in intracellular calcium levels.

Various concentrations of AUR were added to Quin 2/AM-loaded neutrophils (indicated by arrows) and the rises in intracellular calcium levels measured. Control studies indicated that the carrier solvent (0.1% ethanol) did not cause measurable changes in intracellular calcium concentration.


5 min
74.6μM
44.6μM
20.0μM
10.0μM
5.0μM
QUIN 2 FLUORESCENCE INTENSITY

triggered by fMet-Leu-Phe, the increase in calcium concentration proceeded very slowly prior to reaching a plateau. The rate of the calcium rise was proportional to the concentration of AUR utilized. Fura 2/AM-loaded neutrophils were more sensitive to AUR in that the rise in intracellular calcium levels occurred without an apparent lag and reached greater levels of intracellular calcium than that of Quin 2/AM-loaded cells.

The effects observed with AUR were not due to a direct effect of the drug on the fluorescence indicator as studies in which the drug was incubated for extended periods of time with the unesterified form of the indicator (Quin 2), did not reveal any changes in its fluorescence capacity.

The increase in fluorescence was not due simply to leakage of calcium indicator back into the medium, since (a) the addition of EGTA to cell suspensions after AUR exposure, and (b) washing and resuspending AUR-treated cells, did not reverse the rise in fluorescence.

Similar studies carried out with both Quin 2/AM- and Fura 2/AM-loaded cells incubated in the presence of GST, showed that this drug did not act like AUR. Cell suspensions incubated with 50 - 500 μM GST, and continuously monitored for 60 min, showed no fluorescence changes.

When the calcium in the extracellular fluid was

chelated by EGTA, the addition of AUR to the Fura 2/AM-loaded cell suspension produced a very small initial rise in intracellular calcium (Figure 20). Calculations indicate a two-fold increase of intracellular free calcium to approximately 200 nM. This initial rise may represent the mobilization of calcium from internal stores since it is insensitive to the removal of extracellular calcium. However, the major portion of the AUR-induced response is dependent on the presence of calcium in the extracellular fluid.

The extent of AUR-induced changes in intracellular calcium concentration was comparable to that mediated by the calcium ionophores A23187 and ionomycin (Figure 21). Under conditions where neutrophils were treated with the calcium ionophores, the addition of 2 mM EGTA, at peak intracellular calcium levels, precipitated a slow decline in the fluorescence signal, presumably due to efflux of calcium from the cell and dissociation of calcium from Quin 2. In contrast, EGTA added to cells after AUR exposure, had no effect on the elevated increase in intracellular calcium concentration.

J. MEASUREMENT OF PROTEIN KINASE C ACTIVITY IN PHORBOL ESTER-STIMULATED NEUTROPHILS.

Protein kinase activity, specific to protein kinase C, was measured by the ability of protein kinase C to

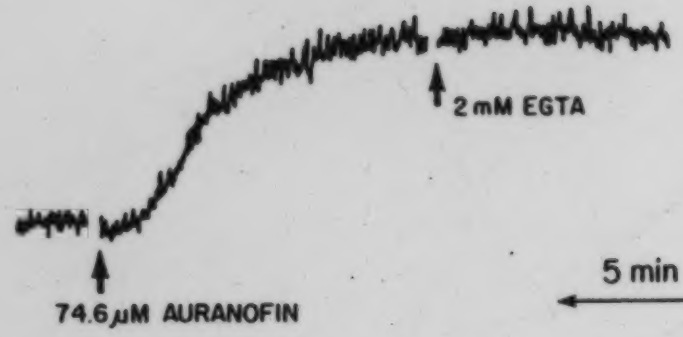
Figure 20: Auranofin-induced changes in intracellular calcium levels in the presence and absence of external calcium.

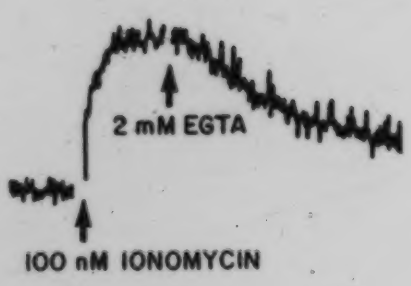
Intracellular calcium levels were monitored by use of the fluorescent calcium probe Fura 2/AM. Auranofin (45 μM), with and without EGTA (2 mM), was added to loaded cell suspensions at times indicated by the arrows.

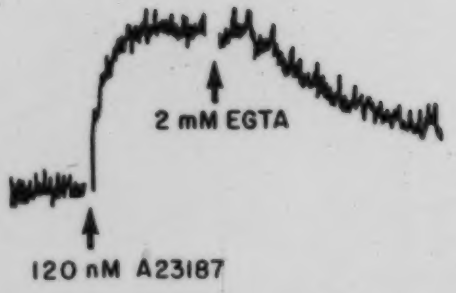
FURA 2 FLUORESCENCE INTENSITY
AUR
AUR + EGTA
1 min

Figure 21: Auranofin- and ionophore-induced changes in intracellular calcium and the effect of EGTA.

Auranofin (74.6 μM), ionomycin (100 nM) and A23187 (120 nM) were added to Quin 2/AM-loaded neutrophils at times indicated by the arrows. EGTA (2 mM) was added to each of the reaction mixtures upon reaching maximum levels of intracellular calcium.


2 mM EGTA
74.6 μM AURANOFIN
5 min


2 mM EGTA
100 nM IONOMYCIN


2 mM EGTA
120 nM A23187

phosphorylate exogenous histone III-S, one of its substrates, in a calcium- and phospholipid-dependent manner. Figure 22 (A) demonstrates that optimum histone phosphorylation was detected when calcium, phosphatidylserine and diolein were present in the assay mixture. Enhanced incorporation of ^{32}P peaked at 10 min and leveled off thereafter. When phosphatidylserine and diolein were removed from the system, a small but significant level of histone phosphorylation was detected, probably due to the activation of other calcium-dependent kinases. Thus, background readings in the presence of calcium alone were utilized as negative controls and were subtracted from positive controls and samples (in the presence of phospholipid and calcium), in all subsequent assays.

Of particular importance is the requirement for leupeptin in the cell homogenization buffer for optimum detection of protein kinase C in subcellular fractions. Leupeptin is an inhibitor of calpain and similar proteases. As was mentioned in the INTRODUCTION, these proteases attack protein kinase C and render it a calcium- and phospholipid-independent kinase. Figure 22 (B) demonstrates that when cells were homogenized in the absence of leupeptin, no calcium- and phospholipid-dependency was observed. Subsequent experiments employed leupeptin in the cell homogenization buffer to

Figure 22: Optimum conditions for detection of protein kinase C activity.

Cytosolic neutrophil fractions were obtained from the 100,000 x g supernate of sonicated resting neutrophils in the presence (A) and absence (B) of 0.1 mM leupeptin as described in MATERIALS AND METHODS. Kinase activity was assayed by measuring the amount of ^{32}P incorporation into exogenous histone over various time intervals up to 10 min. The assay mixture contained 20 mM Tris HCl, pH 7.4; 10 mM $MgCl_2$; 0.2 mM $CaCl_2$; 0.36 mg/mL histone Type III-S; 20 µM [γ -^{32}P]ATP (50 - 100 cpm/pmol); 0.01% Triton X-100; with (○) and without (●) 1.2 mM phosphatidylserine and 15.4 µM diolein; and 50 µL of sample fraction in total volume of 250 µL. Each point in A and B represents the mean of six determinations with less than 5% standard error of the mean.

PROTEIN KINASE C ACTIVITY
(nmol Pi/mg protein)
20
15
10
5
0
0
2
4
6
8
10
TIME (min)
A
B

avoid conversion of protein kinase C to protein kinase M (Melloni et al., 1985), and an incubation period of 15 min for optimal detection of histone phosphorylation in the actual assay.

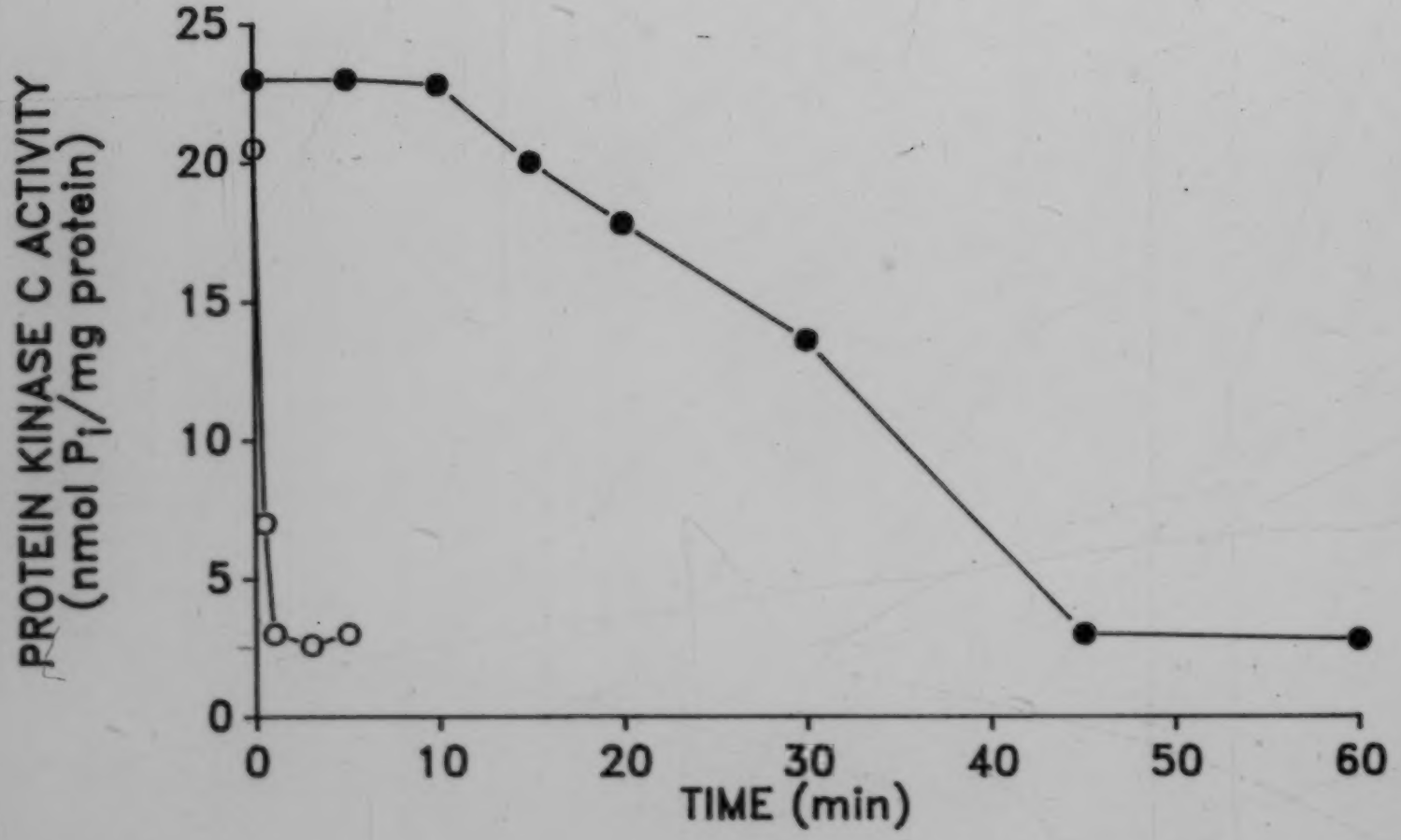
In agreement with previous studies (Pontremoli et al., 1986; Shoji et al., 1986), TPA caused a rapid translocation of protein kinase C from the cytosolic to the particulate fraction of the neutrophil within minutes after its addition to cells. Figure 23 demonstrates the rapid disappearance of cytosolic protein kinase C after the addition of 0.1 μM TPA to cells. Redistribution of protein kinase C by TPA was dose-dependent in that low concentrations (0.1 - 1 nM), although promoting translocation of the kinase, did so at a slower rate than higher concentrations (10 - 100 nM).

K. THE EFFECT OF GOLD COMPOUNDS ON THE DISTRIBUTION OF PROTEIN KINASE C BETWEEN NEUTROPHIL CYTOSOLIC AND PARTICULATE FRACTIONS.

Pretreatment of neutrophils with AUR (20 μM) caused a time-dependent decrease in the amount of protein kinase C activity detectable in the cytosolic fraction (Figure 23). Compared to TPA, the AUR-induced decrease in activity was much more gradual reaching baseline levels between 45 and 60 min. GST (100 μM) caused a

Figure 23: The presence of cytosolic protein kinase C activity in phorbol ester- and auranofin-treated cells.

Neutrophils were treated with TPA (○ , 0.1 μM) or AUR (● , 20 μM) for the indicated periods of time prior to cellular disruption and ultracentrifugation. The 100,000 x g supernatant was assayed for calcium- and phospholipid-dependent kinase activity as described in MATERIALS AND METHODS. The results represent 1 of 5 experiments which provided identical results.

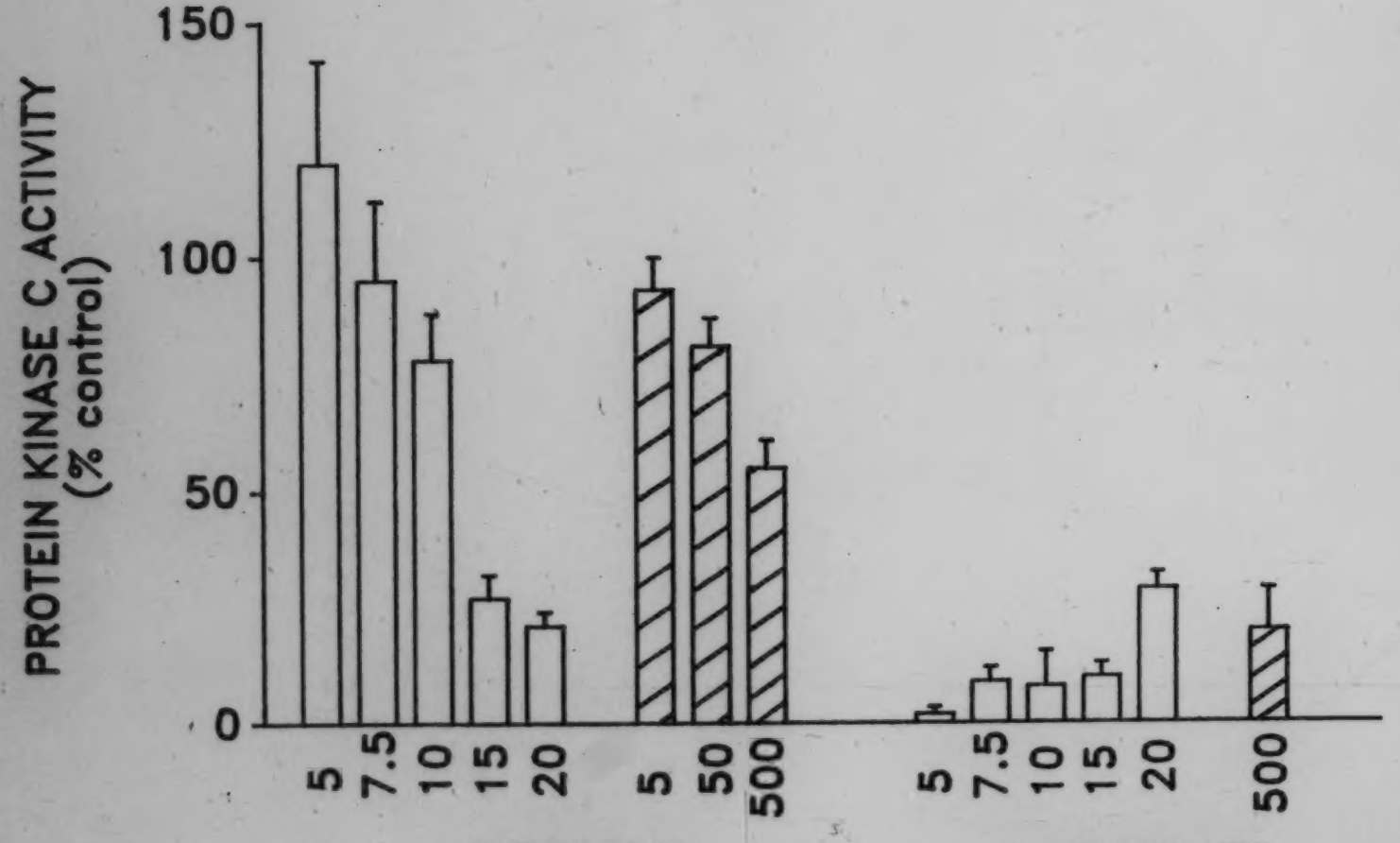

PROTEIN KINASE C ACTIVITY
(nmol P_i/mg protein)
25
20
15
10
5
0
0
10
20
30
40
50
60
TIME (min)

similar decrease in cytosolic protein kinase C activity to that of AUR.

To ascertain whether the gold compounds promoted translocation or redistribution of protein kinase C, particulate and cytosolic neutrophil fractions were assayed for their content of protein kinase C activity (Figure 24). These fractions were obtained from cells which had been treated for 30 min with various concentrations of AUR (5 - 20 μM) or GST (5 - 500 μM). AUR caused a dose-dependent decrease in the levels of cytosolic protein kinase C upon incubation with intact neutrophils. The decline in activity was significant at AUR concentrations greater than 10 μM with increasing concentrations causing decreasing protein kinase C levels. Similar results were obtained with GST but to a lesser extent than AUR. In fact, at equivalent gold concentrations, AUR was at least 5 - 10 times more effective than GST in altering cytosolic protein kinase C levels. The particulate material did not provide evidence of a concomitant increase in protein kinase C levels to match that lost from corresponding cytosolic fractions. A correlation could be made for 20 μM AUR, although the increase in particulate activity was not large enough to account for the loss in kinase activity from the cytosol. For example, 20 μM AUR reduced cytosolic activity by 16.25 ± 2.00 nmol (SE) P_i/ mg

Figure 24: The effects of gold compounds on the distribution of neutrophil protein kinase C activity.

Cytosolic and particulate cell fractions were obtained from the 100,000 x g supernatant and detergent- solubilized pellet of AUR (□ , 5-20 μM) and GST (▨ , 5-500 μM), pretreated cells (37°C, 45 min). Protein kinase C activity was measured as the calcium- and phospholipid-dependent incorporation of ^{32}P onto histone and expressed as a percentage of levels of activity found in cells not exposed to the gold compounds (control). Results are the mean ± standard error of six determinations.

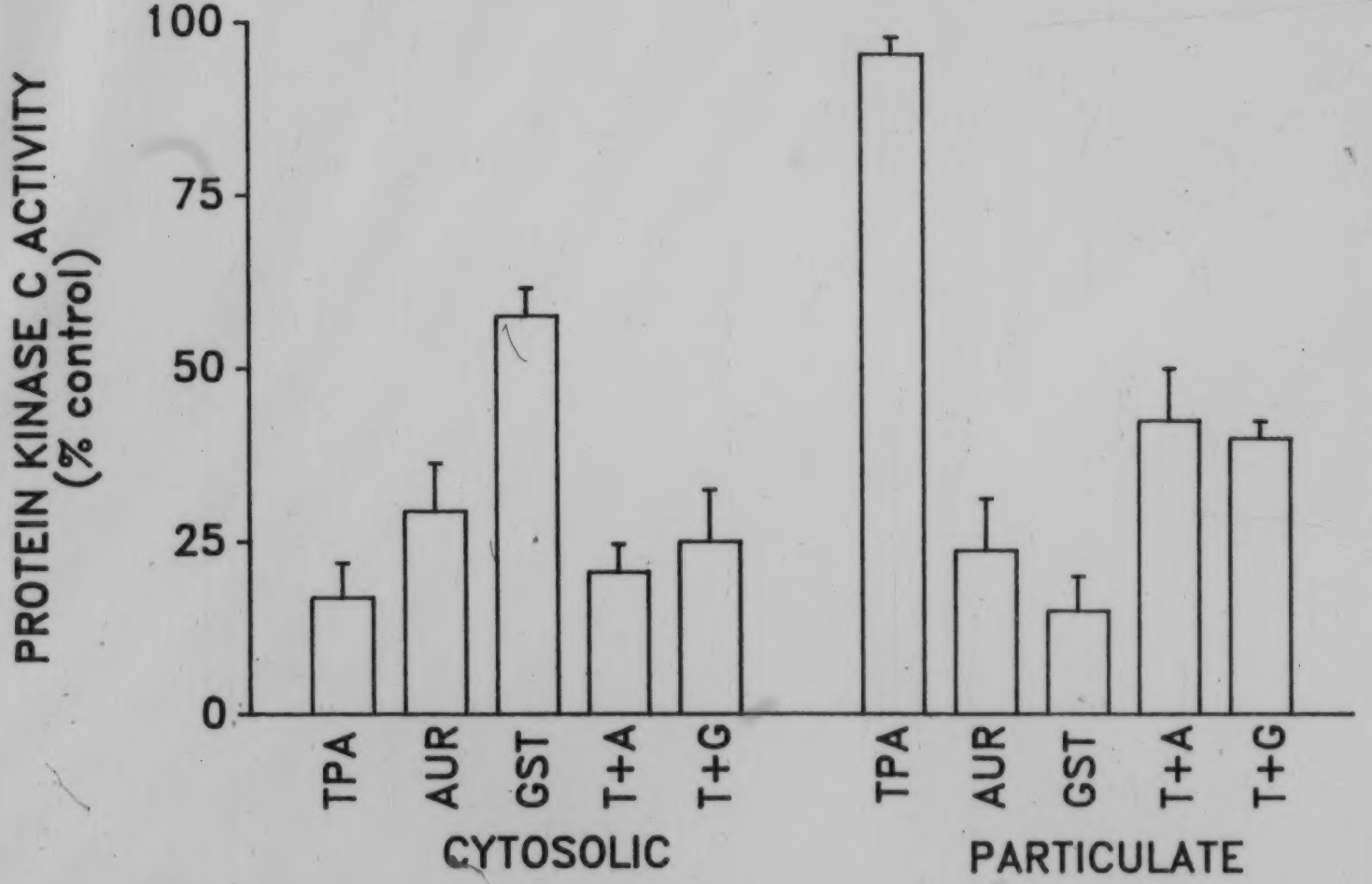

PROTEIN KINASE C ACTIVITY
(% control)
150
100
50
0
5
7.5
10
15
20
5
50
500
5
7.5
10
15
20
500
CYTOSOLIC
PARTICULATE

protein, while increasing the particulate activity by 1.62 ± 0.25 nmol P_i/mg protein. Relative to cytosolic and particulate protein kinase C levels in resting cells, the former declined by 80% while the latter increased 25%. By comparison, TPA decreased cytosolic protein kinase C activity by approximately 85% and concomitantly raised the particulate activity by a similar amount (Figure 25). Under conditions in which TPA was added to cells after a 30 min preincubation with either gold compound, significant changes in cytosolic kinase activity were not observed, and only small increases in activity were detected in the particulate material compared to that measured with TPA alone (Figure 25).

These results can be interpreted in at least two ways. The first is that the gold compounds are directly inhibiting protein kinase C and that the interaction of either drug with this enzyme precludes its activation and redistribution by TPA or diacylglycerol. Alternatively, the gold compounds could promote the translocation of protein kinase C to the membrane and render it inactive either by bound drug or proteolytic damage as a result of its juxtaposition with proteases.

Figure 25: The effects of gold compounds on the phorbol ester-induced distribution of neutrophil protein kinase C.

Cells were pretreated with AUR (20 μM), GST (0.5 mM) or carrier solvent (0.1% ethanol), for 45 min at 37°C. Where indicated, TPA (0.1 μM) was added to cell suspensions at the 35 min time point. The neutrophil fractions were obtained and assayed for calcium- and phospholipid-dependent kinase activity as described in MATERIALS AND METHODS. Results are the mean ± standard error of 5 determinations and are expressed as a percentage of control levels of activity in subcellular fractions present in resting cells.

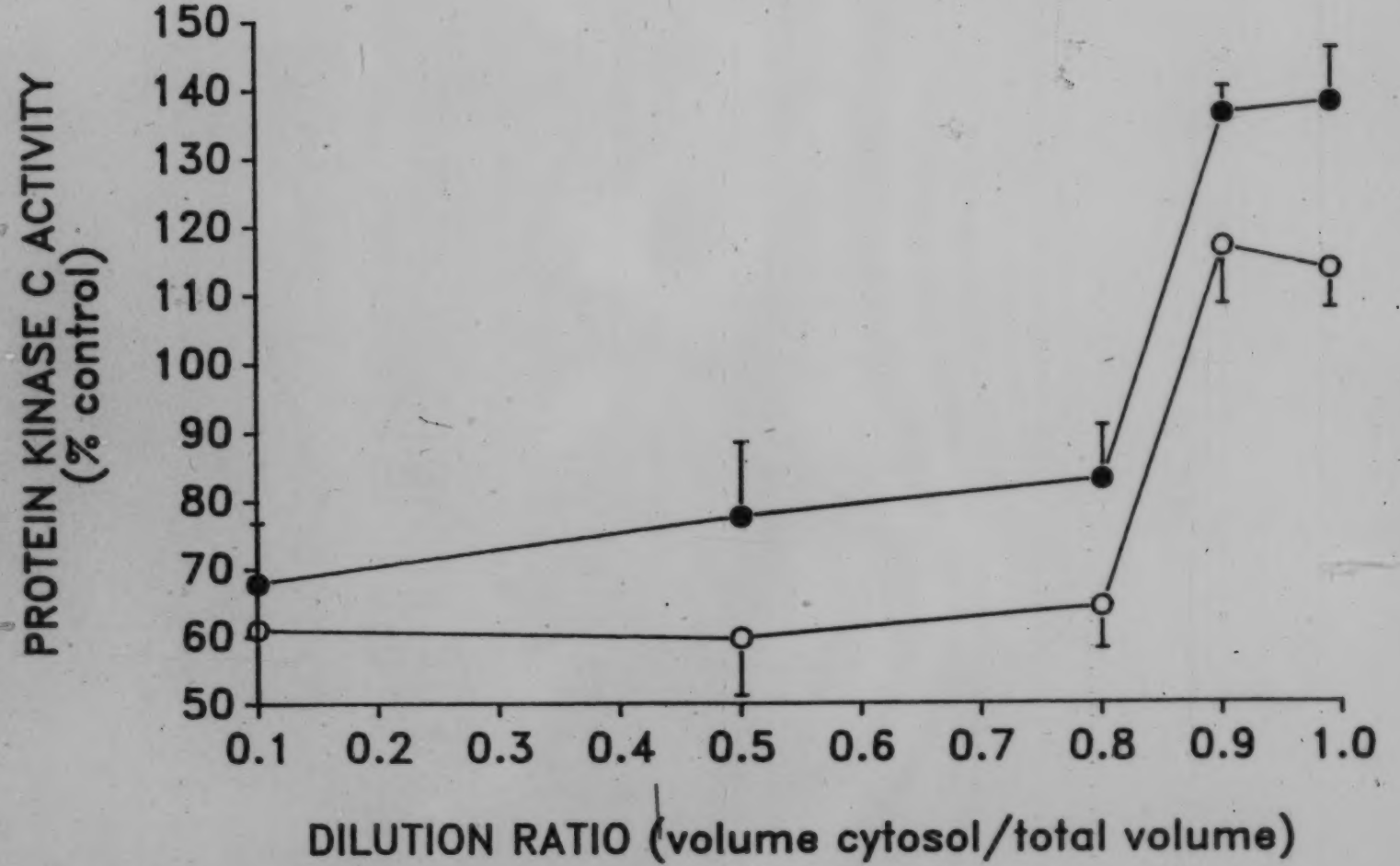

PROTEIN KINASE C ACTIVITY
(% control)
100
75
50
25
0
TPA
AUR
GST
T+A
T+G
CYTOSOLIC
TPA
AUR
GST
T+A
T+G
PARTICULATE

L. NEUTROPHIL CYTOSOLIC AND PARTIALLY-PURIFIED PLATELET PROTEIN KINASE C ACTIVITIES IN THE PRESENCE OF GOLD COMPOUNDS.

This study examined the possibility that the gold compounds may be inhibiting protein kinase C *in situ*. Cytosolic material was obtained from resting cells and was then incubated for various times in the presence of AUR or GST prior to measuring the activity of protein kinase C. Calcium- and phospholipid-dependent kinase activity measured in the cytosol was unaffected by the gold compounds regardless of the concentration of drug employed (0.1 - 100 μM), or the time of incubation (5 - 30 min). Since the cytosolic protein kinase C concentration was unknown, it was assumed to be high and therefore insensitive to a direct action of the gold compounds. Thus, dilutions of the cytosol were made and the effects of 20 μM AUR and 20 μM GST on protein kinase C activity were examined. Figure 26 illustrates that sensitivity to inhibition by the gold compounds increased as the cytosolic material became more dilute. Enzyme activity present in dilutions greater than 0.9 (dilution ratio, volume cytosol/total volume) was enhanced by the presence of AUR or GST. When dilutions less than 0.1 were made, control values of protein kinase C activity could not be measured accurately, and therefore, measurements of activity in the presence of

Figure 26: The direct effect of gold compounds on neutrophil cytosolic protein kinase C.

Neutrophil cytosolic material was obtained from the 100,000 x g supernatant of homogenized cells. Dilutions of the cytosol were made in extraction buffer (25 mM Tris HCl, pH 7.4; 1 mM EDTA; 2 mM PMSF; 0.25 M sucrose; 0.1 mM leupeptin; 10 mM ß-mercaptoethanol), and 20 µM AUR (o) or 20 µM GST (●) were added, mixed and assayed 10 min later, for protein kinase C activity. Each measurement of activity was compared to a control in which the same dilution of cytosol was made but no drug was added. Results are the mean ± standard error of 4 determinations and are expressed in terms of a dilution ratio, e.g. a dilution ratio of 0.5 indicates a 1:1 dilution of cytosol in extraction buffer.


PROTEIN KINASE C ACTIVITY
(% control)
150
140
130
120
110
100
90
80
70
60
50
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
DILUTION RATIO (volume cytosol/total volume)

gold compounds could not be assessed.

A direct effect of the gold compounds on the kinase was further studied by examining the activity of partially-purified protein kinase in the presence of AUR and GST. Both AUR and GST inhibited the activity of platelet protein kinase C in a dose-dependent manner (Figure 27). The estimated concentration giving 50% inhibition (IC_{50}) of AUR was 0.77 μM and that for GST was 3.76 μM. The gold compounds completely inhibited protein kinase C activity at concentrations greater than 10^{-5}M. At a fixed concentration of either AUR or GST (10 μM), inhibition of the enzyme occured instantaneously as it displayed no measurable time-dependence. The carrier solvent, 0.1% ethanol, had no effect on the enzyme compared to control activity.

Inhibition by the gold drugs of the enzyme could be reversed by increasing the concentration of platelet protein kinase C at a fixed AUR concentration under standard assay conditions (Figure 28). This result implies a direct inhibitory effect of the drug on the enzyme.

M. PROTEIN KINASE C-MEDIATED ENDOGENOUS PROTEIN PHOSPHORYLATION IN GOLD COMPOUND-TREATED NEUTROPHILS.

Blockade of protein kinase C activity in intact

Figure 27: The dose-dependent inhibition of platelet protein kinase C by gold compounds.

Platelet protein kinase C was partially purified according to MATERIALS AND METHODS. The initial rate of histone phosphorylation in the absence of gold compounds was 53.2 pmol P_i/min. AUR (o) or GST (●) were added directly to the enzyme prior to assay. Data are the average of duplicate samples from one experiment representative of three providing similar results.

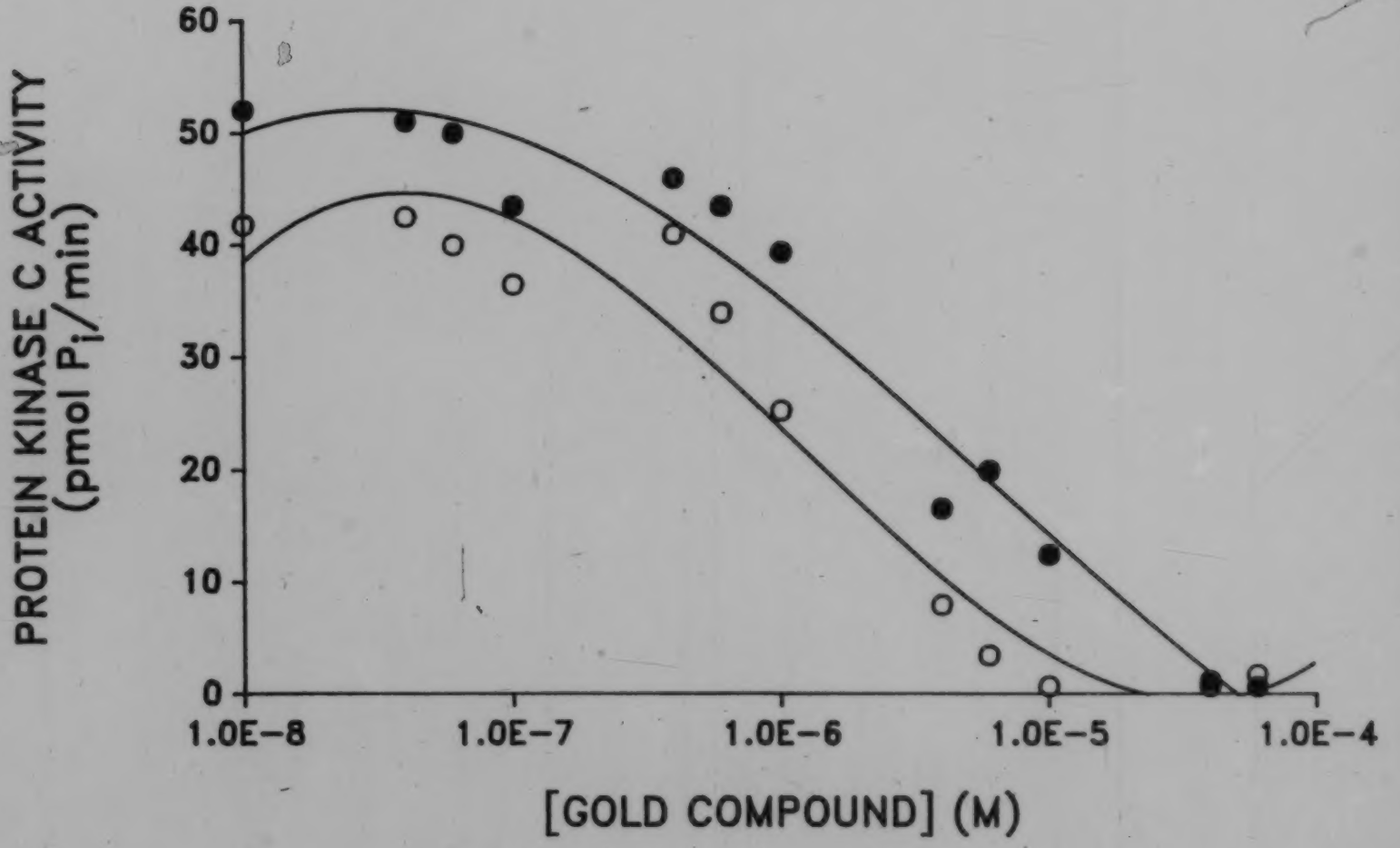
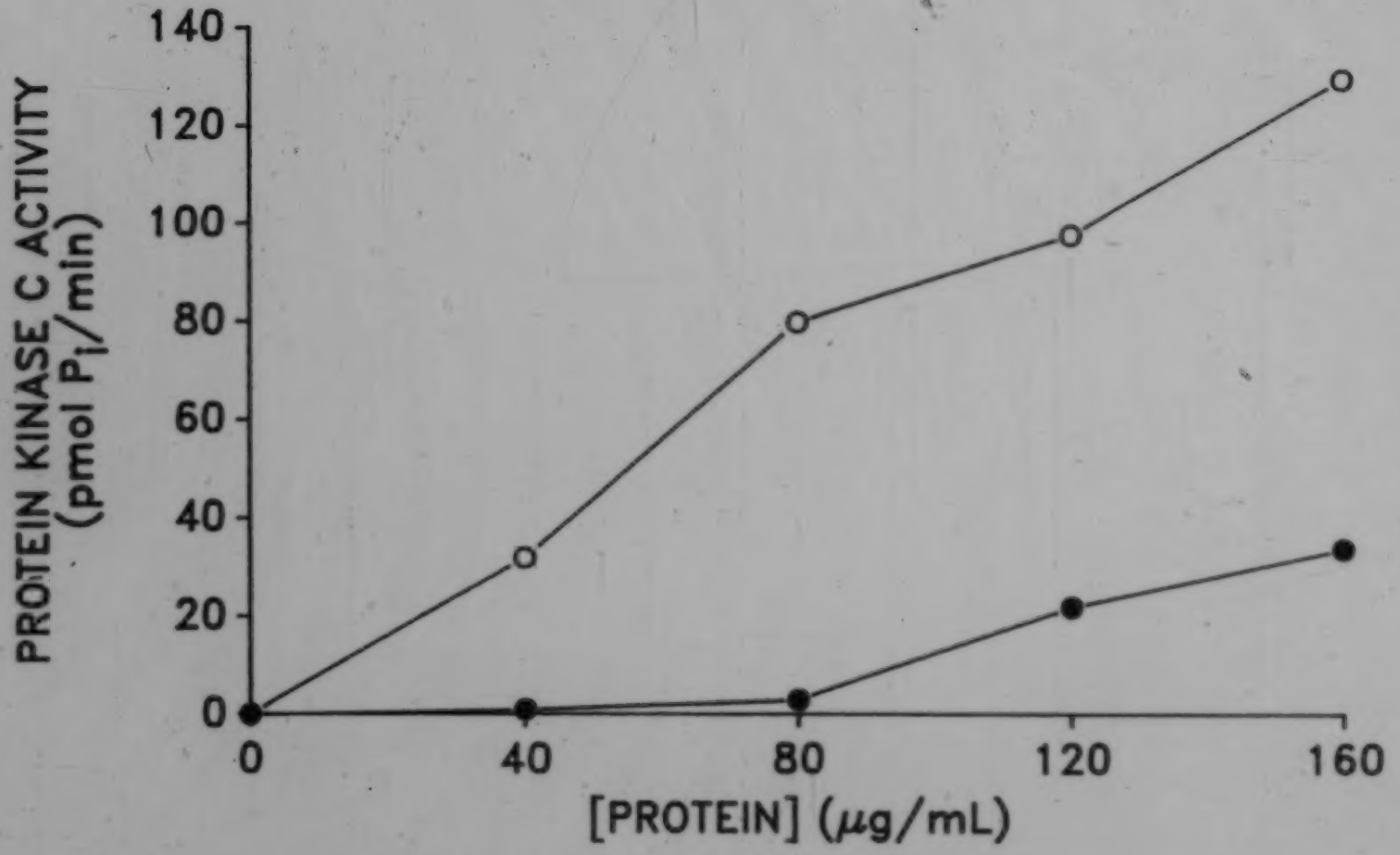

PROTEIN KINASE C ACTIVITY
(pmol P_i/min)
60
50
40
30
20
10
0
1.0E−8
1.0E−7
1.0E−6
1.0E−5
1.0E−4
[GOLD COMPOUND] (M)

Figure 28: The effects of increasing platelet protein kinase C concentration on auranofin inhibition of enzymatic activity.

AUR (● , 10 μM) was added to various concentrations of partially-purified protein kinase C (refer to MATERIALS AND METHODS for details of purification), prior to assay. Corresponding assays were performed in the absence of drug (○ , 0.1% ethanol). Results are the average of duplicate samples of one experiment representative of three providing similar results.


PROTEIN KINASE C ACTIVITY
(pmol P_i/min)
140
120
100
80
60
40
20
0
0
40
80
120
160
[PROTEIN] (μg/mL)

neutrophils was assessed by comparing endogenous protein phosphorylation patterns in cells treated with combinations of TPA and gold compounds (Figures 29 and 30). In agreement with previous reports (Segal et al., 1985; Heyworth and Segal, 1986; Ohtsuka et al., 1986; Hayakawa et al., 1986), a range of proteins were slightly phosphorylated in resting cells. Activation of cells with TPA enhanced phosphorylation of over 10 protein bands which reached maximal levels after a 2 min exposure to the phorbol ester. Proteins of relative molecular mass (M_r) 40,000, 44,000, 47-50,000, 65,000, and 66-90,000 were most heavily labelled.

The addition of 20 μM AUR to labelled cell suspensions, resulted in weaker phosphorylation of proteins compared to that observed with TPA (Figure 29). The exception was a protein of M_r = 20-22,000 which was predominantly phosphorylated after a 2 min incubation with this drug. The simultaneous addition of TPA and AUR to labelled cells resulted in rapid phosphorylation of proteins similar to that observed with TPA alone. With continued incubation, the extent of protein phosphorylation declined to levels close to that observed in the control situation (0.1% ethanol + 0.1% Me_2SO).

Decreased labelling was noted for the M_r = 47-50,000 protein band. The phosphorylation of this protein band has been shown to be protein kinase

Figure 29: Endogenous protein phosphorylation mediated by phorbol ester and auranofin.

Neutrophils were labelled with $^{32}PO_4$ according to MATERIALS AND METHODS. Cells were stimulated with TPA (0.1 μM), AUR (20 μM) or a combination of both, for the times indicated as follows: a - 0.1% ethanol + 0.1% Me_2SO, 2 min; b - TPA, 2 min; c - TPA, 5 min; d - TPA, 10 min; e - TPA 20 min; f - AUR, 2 min; g - AUR, 5 min; h - AUR, 10 min; i - AUR, 20 min; j - TPA + AUR, 2 min; k - TPA + AUR, 5 min; l - TPA + AUR, 10 min; m - TPA + AUR, 20 min; n - 0.1% ethanol + 0.1% Me_2SO, 20 min. This experiment represents one of 4 experiments demonstrating similar phosphorylation patterns.

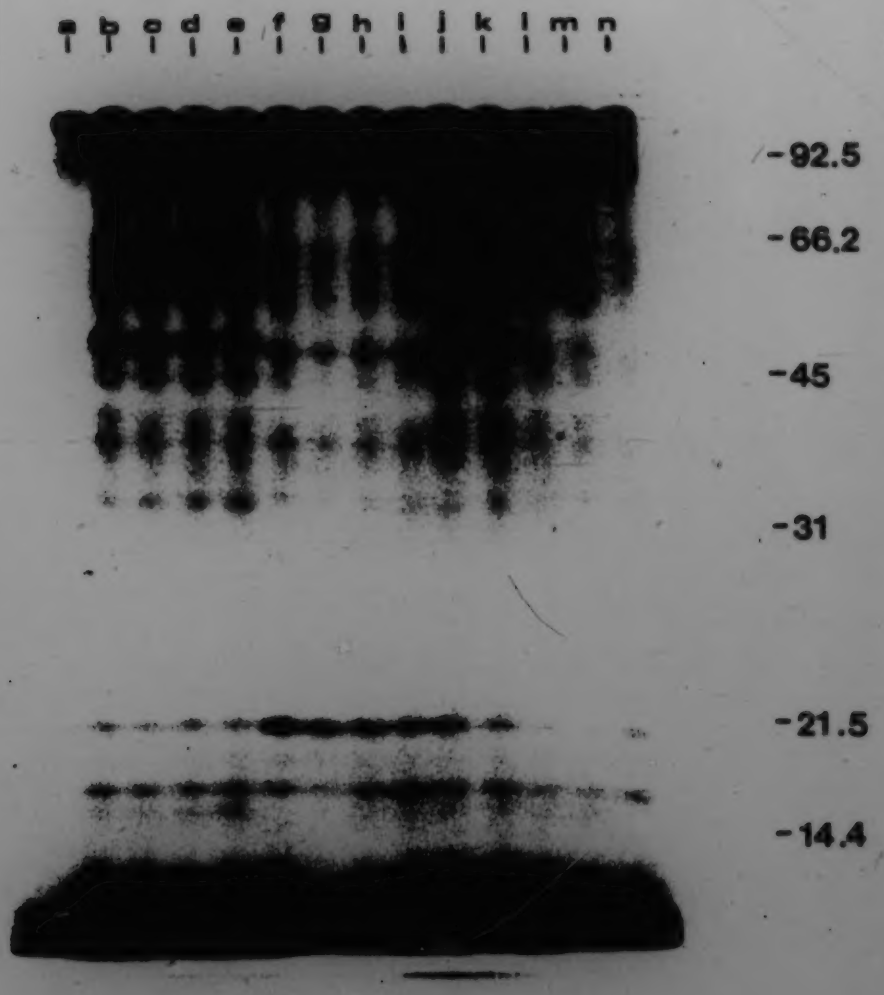
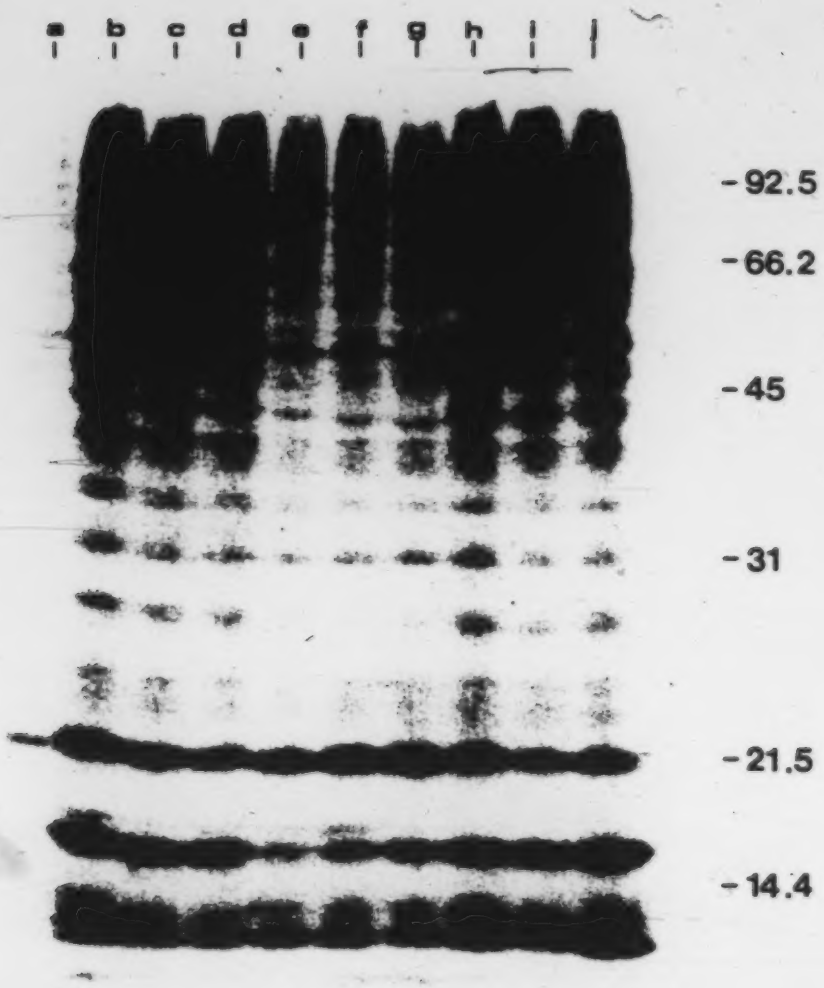

a b c d e f g h i j k l m n
-92.5
-66.2
-45
-31
-21.5
-14.4

Figure 30: Endogenous protein phosphorylation mediated by phorbol ester and gold sodium thiomalate.

Neutrophils were labelled with $^{32}PO_4$ according to MATERIALS AND METHODS. Cells were stimulated with TPA (0.1 μM), GST (20 μM), or a combination of both for the times indicated as follows: a - 0.1% Me_2SO, 2 min; b - TPA, 2 min; c - TPA, 5 min; d - TPA, 15 min; e - GST, 2 min; f - GST, 5 min; g - GST, 15 min; h - TPA + GST, 2 min; i - TPA + GST, 5 min; j - TPA + GST, 15 min. This experiment respresents one of 3 experiments demonstrating similar phosphorylation patterns.


a
b
c
d
e
f
g
h
i
j
-92.5
-66.2
-45
-31
-21.5
-14.4

C-dependent and necessary for the activation of NADPH oxidase, the enzymatic system responsible for the respiratory burst. Studies repeated using GST (20 μM), failed to demonstrate inhibition of TPA-induced protein phosphorylation (Figure 30). However, GST did induce a rapid phosphorylation of the M_r = 20-22,000 protein similar to AUR.

Forskolin, a known activator of adenylate cyclase, and therefore, of the cyclic adenosine monophosphate-dependent protein kinase, induced protein phosphorylation in neutrophils which was insensitive to the presence of AUR or GST. Similarly, neither gold compound affected protein phosphorylation induced by an increase in intracellular calcium levels, that is, in cells stimulated by the calcium ionophore, A23187.

N. GOLD COMPOUND-INDUCED DISPLACEMENT OF PHORBOL ESTER FROM NEUTROPHIL CYTOSOLIC AND PARTIALLY-PURIFIED PLATELET PROTEIN KINASE C.

The ability of the gold compounds AUR and GST, to displace phorbol 12,13-dibutyrate from its cellular receptor, protein kinase C, was examined in this study. Phorbol 12,13-dibutyrate is a phorbol ester which displays a ten-fold lower potency and much less liphophilicity than TPA. Due to its extensive nonspecific interactions with cellular components, [^{3}H]TPA has

been replaced by [^{3}H]phorbol 12,13-dibutyrate in assays of binding to protein kinase C. The specific binding of [^{3}H]phorbol 12,13-dibutyrate was found to be saturable, of high affinity, specific for biologically active phorbol esters, reversible and stable (Delclos et al., 1980; Driedger and Blumberg, 1980).

Specific binding of [^{3}H]phorbol 12,13-dibutyrate to the neutrophil cytosolic receptor was dependent upon the presence of phosphatidylserine and calcium in the reaction. It could be displaced from the receptor by the addition of increasing concentrations of diolein or TPA. Binding of the ligand to the cytosol increased with time of incubation at 37°C and reached a plateau at 15 min. A Scatchard analysis of [^{3}H]phorbol 12,13-dibutyrate binding to the neutrophil cytosolic receptor is shown in Figure 31. A dissociation constant for phorbol 12,13-dibutryate from the receptor (K_D) was found to be 9.9 nM, a value consistent with previous reports (5-90 nM, Sharkey et al., 1984; König et al., 1985).

When specific binding of [^{3}H]phorbol 12,13-dibutyrate to its cytosolic receptor was measured in the presence of either AUR or GST, a dose-dependent inhibition was observed (Figure 32). Neither drug affected nonspecific binding. IC_{50} values were 75 μM

Figure 31: Scatchard analysis of specific [^{3}H]phorbol 12,13-dibutyrate binding to the neutrophil cytosolic receptor.

Specific binding to the neutrophil cytosolic receptor was measured in the presence of phosphatidylserine (62.5 µg/mL), calcium chloride (1 mM), phorbol 12,13-dibutyrate (30 µM) and a range of [^{3}H]phorbol 12,13-dibutyrate concentrations. Reaction mixtures were incubated at 37°C for 15 min. The nonlinear regression program LIGAND was used for curve fitting. Points are the average of triplicate determinations.

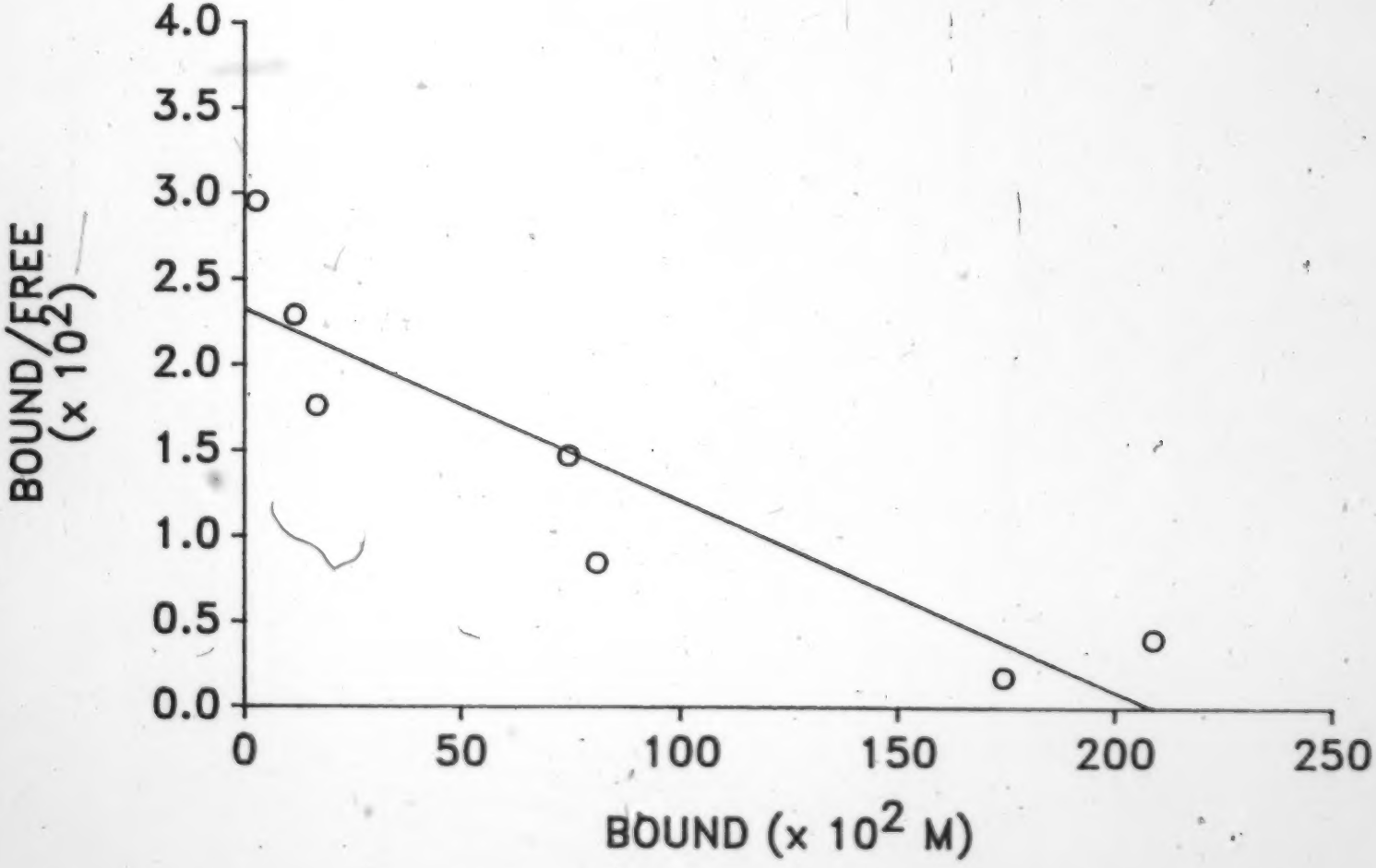
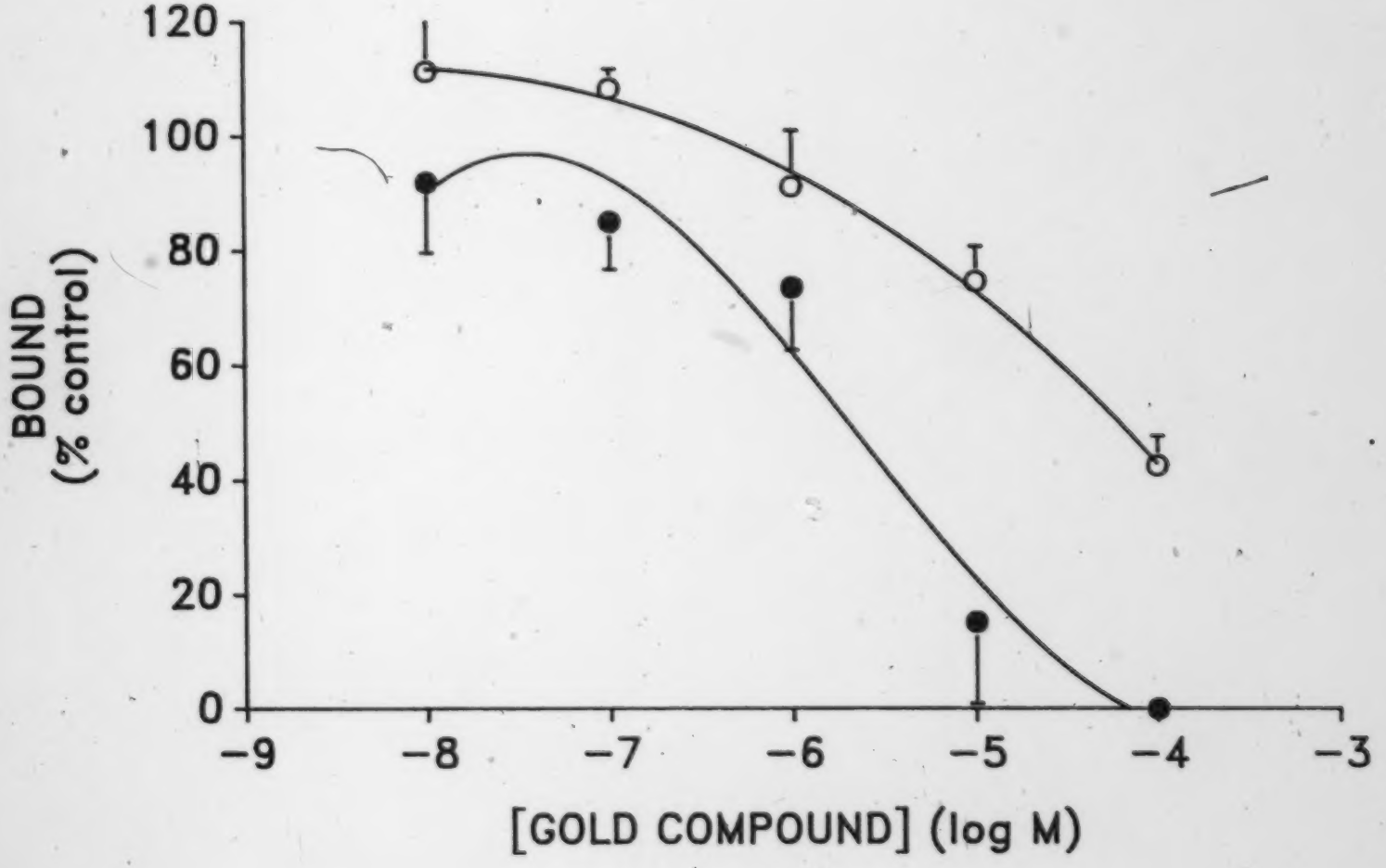

BOUND/FREE
(x 10²)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
BOUND (x 10² M)

Figure 32: Inhibition of specific [^{3}H]phorbol 12,13-dibutyrate binding to the neutrophil cytosolic receptor by auranofin and gold sodium thiomalate.

Specific binding of [^{3}H]phorbol 12,13-dibutyrate was measured in the presence of phosphatidylserine (62.5 µg/mL), calcium chloride (1 mM), and the indicated concentrations of gold compounds to approximately 3 mg/mL cytosolic protein. The mixture was incubated for 15 min at 37°C. Nonspecific binding was measured with determinations at each drug concentration. A final concentration of 10 nM hot ligand and 1 µM cold ligand were utilized. Each point represents the mean ± standard error of five experiments. AUR (○), GST (●).


120
100
80
60
40
20
0
BOUND
(% control)
−9
−8
−7
−6
−5
−4
−3
[GOLD COMPOUND] (log M)

and 2.5 μM for AUR and GST, respectively. These values correspond to approximate inhibitory constants (K_i) values of 37 μM for AUR and 1.2 μM for GST using the equation $K_i = IC_{50}/(1 + 1/K_d)$ as described in MATERIALS AND METHODS.

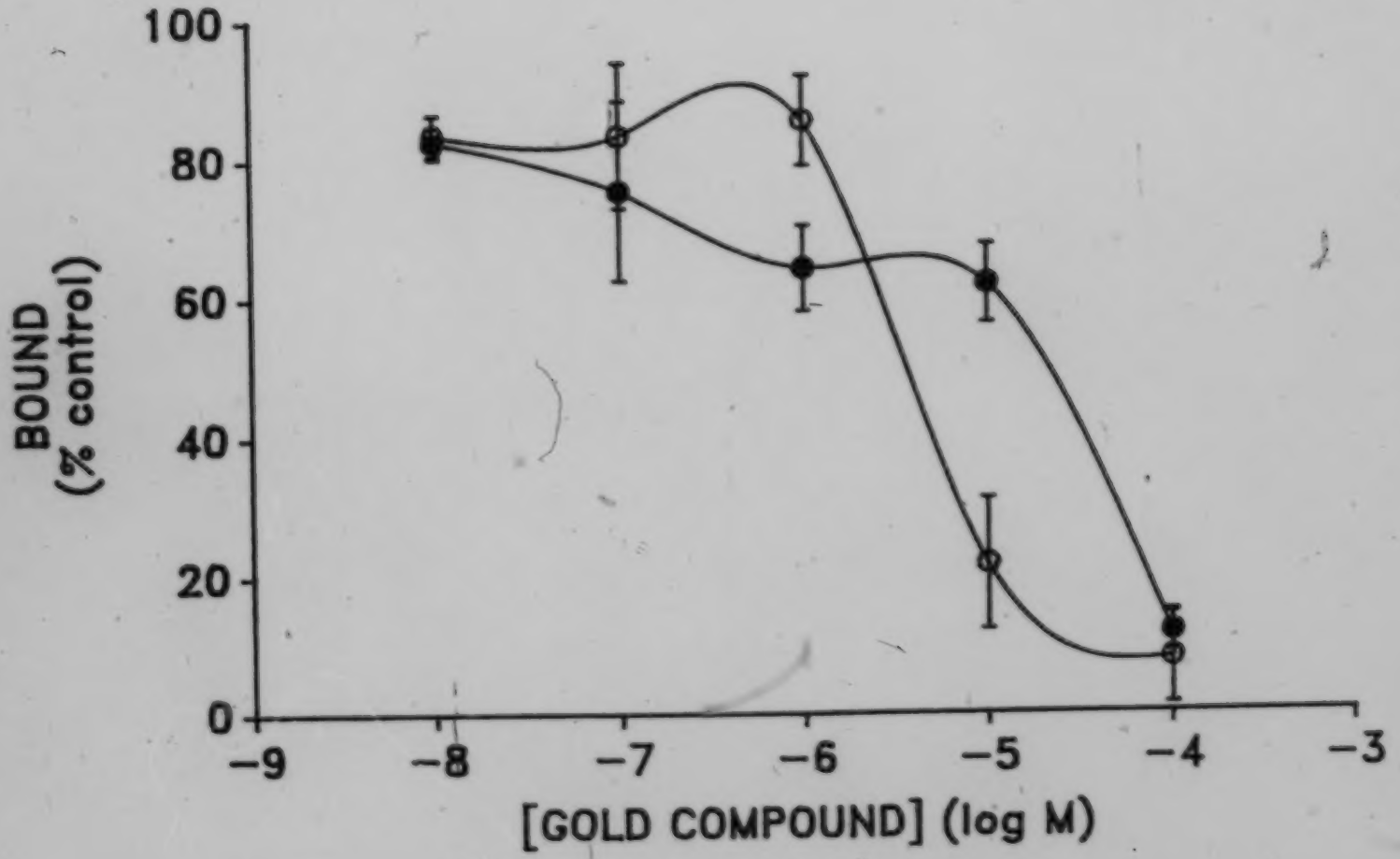
Similar results were obtained when specific binding of [^{3}H]phorbol 12,13-dibutyrate to partially-purified platelet protein kinase C was measured. Both gold compounds displaced bound ligand in a biphasic manner. GST appeared to be more effective in displacing the ligand from its receptor (Figure 33), since the IC_{50} of GST (4 μM) was approximately five-fold less than that for AUR (22 μM).

O. IMMUNOBLOT ANALYSIS OF THE INTEGRITY AND DISTRIBUTION OF NEUTROPHIL PROTEIN KINASE C IN THE PRESENCE OF GOLD COMPOUNDS.

Polyclonal antibodies raised against rat brain protein kinase C have been utilized to measure levels of this enzyme in various tissues, cells and cellular fractions (Girard et al., 1985; Girard et al., 1986; Huang et al., 1986; Girard et al., 1987). In the majority of the cells studied, one of the major proteins detected by immunoblot analysis has a molecular weight of approximately 80,000, characteristic of native protein kinase C. Alterations in the size of the enzyme

Figure 33: Inhibition of specific [^{3}H]phorbol 12,13-dibutyrate binding to the partially-purified platelet receptor by auranofin and gold sodium thiomalate.

Specific binding of [^{3}H]phorbol 12,13-dibutyrate was measured in the presence of phosphatidylserine (62.5 µg/mL), calcium chloride (1 mM), and the indicated concentrations of gold compounds to approximately 0.3 mg/mL partially-purified platelet protein kinase C. An incubation period of 15 min at 37°C was employed. Nonspecific binding was measured with determinations at each drug concentration. A final concentration of 10 nM hot ligand and 1 µM cold ligand were utilized. Each point represents the mean ± standard error of values from four experiments. AUR (○), GST (●).


100
80
60
40
20
0
BOUND
(% control)
−9
−8
−7
−6
−5
−4
−3
[GOLD COMPOUND] (log M)

due to proteolytic enzyme activity can also be detected since the antibodies can recognize various epitopes of the molecule. Thus, immunoreactive fragments of 50,000 and 67,000 molecular weight have been detected, among others, and shown to be derived from the native enzyme.

In the present studies, cells pretreated with carrier solvent, TPA, AUR, or GST, for 15 min, were homogenized in hot SDS buffer to prevent proteolysis of the native protein kinase C species (Girard et al., 1986). Immunoblot analyses of the homogenates were performed to investigate whether the gold compounds promoted proteolytic degradation of protein kinase C. This may account for the gold drug-induced reduction in enzyme activity detected in Figures 23 and 24. Figure 34(A) illustrates the pattern of immunoreactive bands under the four conditions examined. The main conclusion is that the gold compounds did not alter the molecular weights of the bands recognized by the antiserum compared to those recognized in control or TPA-treated cells. However, the major immunoreactive band in all of the homogenate samples had a relative molecular weight of approximately 67,000, much less than the native enzyme species. Smaller fragments were also detected but to a lesser extent. Addition of the protease inhibitors PMSF, leupeptin, or diisopropylfluoro-phosphate, did not alter the size of these fragments,

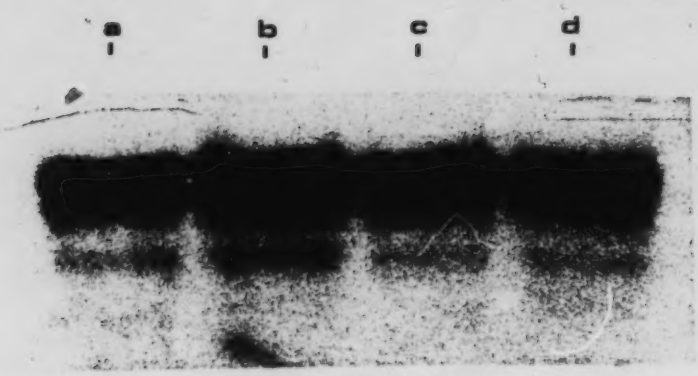
Figure 34: Immunological detection of protein kinase C in neutrophil homogenates, cytosolic and particulate fractions.

Samples were prepared according to MATERIALS AND METHODS. Cells were stimulated with TPA (0.1 µM), AUR (20 µM), GST (0.5 mM), or combinations of these, for 15 min (A) and 5 min (B) at 37°C prior to homogenization and separation into subcellular components. Results are representative of at least one other providing similar blotting patterns.

(A) Whole cell homogenates: a - 0.1% ethanol + 0.1% Me_2SO; b - TPA; c - AUR; d - GST.

(B) Neutrophil cytosolic (c) and particulate (p) fractions: a - 0.1% ethanol + 0.1% Me_2SO; b - TPA; c - AUR; d - GST; e - TPA + AUR; f - TPA + GST.

A



B

that is, native protein kinase C (80,000) could not be detected in the neutrophil homogenates (Dr. Peggy Girard, personal communication).

Figure 25 demonstrates an inhibition of TPA-induced translocation of protein kinase C activity by the gold compounds. To verify these results, immunoblot analyses were conducted on neutrophil cytosolic and particulate fractions under varying conditions of TPA and gold drug pretreatment. The results of Figure 34(B) indicate that TPA caused a pronounced decrease in the major immunoreactive fragment of protein kinase C (67 kD) from the cytosolic fraction of the neutrophil. This decrease was not accompanied by a corresponding increase in the particulate fraction. Girard and coworkers (1987) reported a similar finding in Chinese hamster ovary cells. They found that TPA enhanced degradation of particulate-associated protein kinase C which could be reversed by the addition of diisopropylfluorophosphate. The addition of this protease inhibitor to neutrophils did not reduce degradation of the particulate-associated immunoreactive material (data not shown). Regardless of the duration of TPA pretreatment of the cells, insufficient particulate-associated material was recovered to account for the material lost from the cytosol. Co-treatment of cells with TPA and either AUR (20 μM) or GST (0.5 mM) inhibited the reduction in

cytosolic material observed with stimulation by TPA alone. Thus, the gold compounds interfered with the ability of TPA to reduce immunoreactive material in the cytosolic fraction of the neutrophil. Since this fragment is recognized by the antiserum, it is assumed to be derived from protein kinase C.

To date, these are the first studies in which TPA-induced translocation of protein kinase C in the neutrophil was investigated. The major immunoreactive band was approximately 67 kD and probably represents a proteolytic fragment of the native protein kinase C species. However, this could not be substantiated by studies wherein various protease inhibitors were added to the cell to prevent degradation of the enzyme. It should be noted that the neutrophil is a specialized cell replete with large quantities of proteases whose effects may not be overcome during cell lysis. Whether protein kinase C is normally a 67 kD neutrophil protein or is degraded to this state during experimental manipulation remains to be investigated. This protein does retain calcium- and phospholipid-kinase activity since such activity can be measured in neutrophil cytosolic and particulate fractions.

CHAPTER FOUR

DISCUSSION

I THE LOCALIZATION OF GOLD DRUG ACTION TO PROTEIN KINASE C

The main thrust of this research project was to examine the mechanism(s) of action of gold compounds used therapeutically in the treatment of rheumatoid arthritis. Utilization was made of the neutrophil and the studies focussed on the underlying mechanisms behind the multiple antiinflammatory effects exhibited by AUR and GST. The major conclusion, based on the results of these studies, is that gold coordination complexes interfere with neutrophil signal transduction at the level of protein kinase C.

In the resting cell, the major fraction of protein kinase C activity was localized to the cell cytosol (approximately 20-30 nmol P_i/mg protein). Both AUR and GST induced a decrease in cytosolic enzymatic activity to baseline levels within 45-60 min after their addition to cells. TPA, a known tumour-promoting phorbol ester and activator of protein kinase C, induced a decrease in cytosolic enzymatic activity and a concomitant increase in particulate-associated activity. This process took place within 5 min at a TPA concentration of 0.1 uM, and was commensurate with kinetics of cellular activation. Since the time course of disappearance of cytosolic protein kinase C activity

mediated by the gold compounds was much longer than that of TPA, and since the gold compounds alone were unable to elicit activation of neutrophil functions, it was postulated that the gold compounds were inhibiting protein kinase C activity as opposed to inducing its redistribution to the particulate fraction. In fact, enzyme assays of the cytosolic fractions indicated that, while there was a dose-dependent decrease in cytosolic protein kinase C activity with both AUR and GST, an increase in particulate-associated activity of equal magnitude was not detected (Figure 24). Further evidence was provided by immunoblot analysis of cytosolic neutrophil fractions; the TPA-induced decrease in cytosolic protein kinase C was not observed when the cells were co-stimulated with the gold compounds [Figure 34(B)]. A further point to be made is that if the gold compounds were inhibiting the enzyme this inhibition did not alter its structure since it was still recognized by anti-protein kinase C antibodies.

Direct evidence of protein kinase C inhibition by the gold compounds was obtained by measuring the altered activity of neutrophil cytosolic protein kinase C and partially-purified platelet protein kinase C in the presence of AUR or GST. The neutrophil cytosolic protein kinase C activity in the 100,000 x g supernatant of a 40 x 10^6 cell homogenate, was insensitive to the

presence of either AUR or GST prior to assay. Since the cytosol is a concentrated mixture of many cellular proteins, some which may interfere with the action of the gold compounds, dilutions were made of this material and the activity measured in the presence and absence of either AUR or GST. The gold compounds enhanced the activity of protein kinase C when the dilution ratio was greater than 0.9 (volume cytosol/total volume). Why these small dilutions enhance activity of the enzyme is under investigation. However, dilution ratios less than this became sensitive to inhibition by AUR and GST (Figure 26). Both AUR and GST displayed similar dose-dependent inhibition of platelet protein kinase C (Figure 27). Protein kinase C activity dropped to baseline levels at concentrations of either drug greater than 50 μM. This inhibition was reversed by increasing the concentration of protein in the assay, that is, protein kinase C present in the reaction. Thus, it is concluded that the gold compounds inhibit protein kinase C activity in situ. They do so in a manner dependent on either the amount of protein kinase C or the interfering proteins in the reaction mixture, since inhibition of the enzyme was detected only in a dilute state of the sample under investigation.

Since the gold compounds were shown to directly inhibit protein kinase C in situ, it follows that the

interaction of either AUR or GST with protein kinase C should preclude phorbol ester or endogenous diacylglycerol activation and translocation of the enzyme. In fact, TPA-mediated neutrophil responses were suppressed in cells which were either pretreated or co-stimulated with AUR or GST. Both the TPA-mediated rate of generation and yield of superoxide anion were suppressed in a time- and dose-dependent manner by AUR. Redistribution of neutrophil protein kinase C activity affected by TPA was significantly inhibited by pretreating the cells with either AUR or GST (Figure 25). Within minutes of its addition to cells, TPA induced the phosphorylation of many cellular proteins through its activation of protein kinase C. This response was significantly reduced over time when cells were stimulated by TPA in the presence of AUR. Optimal inhibition was detected between 10 and 20 min. This mode of inhibition was not observed with GST, however, both gold compounds were able to displace the binding of phorbol ester to its neutrophil cytosolic receptor and to partially-purified platelet protein kinase C. In these experiments, GST was more effective that AUR in inhibiting the binding of phorbol 12,13-dibutyrate to protein kinase C. This implies that GST should be a competitive inhibitor of protein kinase C with respect to phorbol ester or diacylglycerol activation. Altera-

tions in binding by AUR may not be a significant factor in its inhibition of protein kinase C, and its mechanism of inhibition may differ from that of GST. Further studies may reveal the significance of these results which include an analysis of enzyme kinetics.

In summary, AUR inhibited all of the phorbol ester-induced responses studied in the neutrophil, whereas, GST produced variable results. In fact, GST displayed inhibitory activity toward protein kinase C-mediated responses only when this drug was directly associated with protein kinase C, that is, in neutrophil cytosolic and platelet protein kinase C preparations. With intact cells, inhibitory activity was not detected unless GST was allowed to incubate with the cells for periods of time in excess of 30 min at 37°C and at concentrations greater than 0.5 mM.

II PROPOSED STRUCTURAL REQUIREMENTS OF GOLD COMPOUNDS FOR OPTIMAL PROTEIN KINASE C INHIBITION

The following model has been developed to explain the possible mechanism whereby the gold compounds could inhibit protein kinase C. Step 1: Cellular uptake of the gold compound by displacement of a sulfur-containing ligand attached to the gold nucleus by cell membrane-associated thiol groups. Step 2: Intracel-

lular distribution of the compound by cytosolically-localized sulfhydryl groups which compete for membrane-bound gold at the former sulfur-containing ligation site. Protein kinase C becomes a candidate for gold complexation and subsequent inhibition at its cysteine-rich region. That the sulfhydryl shuttle mechanism may be utilized by gold compounds to enter cells and inhibit protein kinase C is evidenced by the ability of dithiothreitol to reverse AUR-induced inhibition of the neutrophil respiratory burst. In addition, Snyder and coworkers, (1987) examined the uptake of radiolabelled gold compounds by RAW 264.7 macrophages and demonstrated that cellular association of AUR and its chloro-analogue, triethylphosphine gold chloride (TEPG), was concentration-, time-, and temperature-dependent. The cell associated gold from both drugs increased as a function of time for 10 min and then plateaued between 10 and 30 min. More gold from TEPG associated with cells than from AUR, perhaps due to the greater leaving tendency of the chloride from TEPG which may facilitate interaction with thiols not seen with AUR. Cellular uptake of both gold compounds was inhibited by pretreating cells with N-ethylmaleimide, an irreversible sulfhydryl alkylating agent. Incubation of the gold compounds with glutathione also reduced cellular association and uptake. Thus, interactions of these

compounds with the cell depend solely on the reactivity of the gold and its ligands and the availability of cellular and membrane sulfhydryl groups.

In order for a gold coordination complex to reach protein kinase C and inhibit its activity, a few criteria must be met. The gold compound must contain an active leaving group for gold complexation to occur with membrane-associated thiols. Of equal importance, the other ligand(s) attached to the gold nucleus should permit entrance of the molecule into the lipid bilayer of the plasma membrane. The triethylphosphine (PEt_3) moiety of AUR fulfils this criterion, whereas GST does not possess such a ligand and remains water soluble (Figure 35). This may explain the variable results observed with GST. For example, inhibition of the neutrophil respiratory burst, degranulation, TPA-mediated translocation of protein kinase C and endogenous phosphorylation, required prolonged incubation of GST with the cells. In fact, only slight inhibition was detected at the highest GST concentrations. Although GST may gain entrance into the cell via the sulfhydryl shuttle mechanism, entrance may also occur via phagocytosis of GST polymers at the cell surface, as this compound is more apt to combine with other GST molecules through sulfhydryl bridging. This would account for the prolonged time necessary to

Figure 35: Molecular structures of auranofin, gold sodium thiomalate and D-penicillamine. Shown also is a possible polymeric structure of gold sodium thiomalate.

$R = \begin{matrix} CH\text{-}CO_2\text{-}Na^+ \\ | \\ CH\text{-}CO_2\text{-}Na^+ \end{matrix}$

$Ac = COCH_3$

$Et_3 = (C_2H_5)_3$

(Sadler, 1982)

Page 194 has been removed because of the inavailability of copyright permission. The material included the molecular structures of auranofin, gold sodium thiomalate, D-penicillamine and a polymeric gold compound taken from Sadler, P.J. J. Rheumatol. (suppl. 8)9, 71, 1982.

observe GST-induced alterations in neutrophil responsiveness. When the membrane barrier is removed, GST and AUR are equipotent in inhibiting protein kinase C and displacing phorbol ester binding.

One question which arises in adopting the sulfhydryl shuttle mechanism to the action of gold compounds, is whether the gold atom is a necessary component of the molecule. All of the commonly used gold compounds in the treatment of rheumatoid arthritis contain a sulfur atom and are able to liberate sulfhydryl groups and partake in thiol-exchange reactions (Jellum and Skrede, 1976). Another widely used anti-arthritic agent is D-penicillamine (Figure 35). Although this compound does not contain a gold atom in its molecular structure, it does possess a sulfhydryl group and could, therefore, possibly enter cells and alter intracellular proteins via the sulfhydryl shuttle mechanism (Huck et al., 1984). Thus, D-penicillamine is a good control for testing whether a thiol-containing compound could alter cellular functioning in the absence of a metal ion. In all studies where either AUR, GST, or both, were shown to inhibit neutrophil responsiveness, D-penicillamine had no effect. Neutrophil degranulation, superoxide anion generation, calcium mobilization, protein kinase C activation, endogenous protein phosphorylation, and phorbol 12,13-dibutyrate binding were unaffected by the

presence of D-penicillamine at concentrations ranging from 0.1 - 100 μM. Gold, therefore, does appear to be vital to the inhibitory activities of the gold salts. Further evidence of this comes from the inability of the ligands attached to both AUR and GST to alter any of the neutrophil responses studied (results not shown).

III CONSEQUENCES OF PROTEIN KINASE C INHIBITION

A. EFFECTS ON NEUTROPHIL DEGRANULATION

A possible involvement of protein kinase C in the degranulation of neutrophils has recently been reported (White et al., 1984; O'Flaherty et al., 1984; Robinson et al., 1984; Hoult and Nourshargh, 1985; Kang et al., 1985; Barrowman et al., 1986). In these studies, TPA and 1-oleoyl-2-acetylglycerol were found to promote the release of specific granular components. On the other hand, A23187 (1 μM) induces the release of ß-glucuronidase, a constituent of azurophilic granules. Thus, the system involved in the release of specific granules does not appear to require an increase in intracellular calcium levels, whereas the release of azurophilic granules requires relatively higher intracellular calcium levels. In these granular-releasing systems, it is postulated that substrate proteins with different affinities for protein kinase C are involved and are

phosphorylated to different extents. In the case of the azurophilic granular system, protein kinase C-induced phosphorylation of target proteins is an insufficient signal and enzyme release is observed only in synergism with the calcium signal. However, activation of protein kinase C is sufficient to cause release of specific granular components. Inhibition of protein kinase C would, therefore, alter cellular degranulation from both systems. This is consistent with the finding that AUR inhibited both lysozyme and ß-glucuronidase release from fMet-Leu-Phe-stimulated neutrophils. FMet-Leu-Phe binding to the neutrophil surface induces the formation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol with subsequent mobilization of intracellular calcium stores and activation of protein kinase C. In the presence of AUR, degranulation is suppressed due to the proposed inhibition of protein kinase C. It is interesting to note that the release of granular enzymes induced by A23187 was not inhibited by AUR or GST (Hafström et al., 1983a), presumably because in this situation, degranulation is induced in the absence of protein kinase C activation.

B. THE INVOLVEMENT OF LIPOXYGENASE

Studies of Wey and Baxter (1986), McColl and coworkers (1986), and Liles and coworkers (1987), have

provided evidence for the involvement of protein kinase C in the regulation of the 5-lipoxygenase pathway. The enzyme 5-lipoxygenase catalyzes the initial step in the conversion of arachidonic acid to leukotrienes, a class of potent inflammatory mediators. These groups established that low concentrations of A23187, in combination with either TPA or 1-oleoyl-2-acetylglycerol, synergistically stimulate the de novo production of leukotrienes and their oxidation products. Neither agent alone was able to stimulate leukotriene production. Although the concentration of A23187 employed in these studies (0.1 - 1 μM), did increase intracellular calcium levels, it failed to induce the formation of 5-lipoxygenase products in neutrophils. Thus, a phorbol ester and calcium ionophore can act synergistically to mimic the intracellular events responsible for 5-lipoxygenase activation induced by physiological stimuli. These findings may provide an explanation for the preferential AUR-induced inhibition of leukotriene synthesis, compared to prostaglandin synthesis, in the fMet-Leu-Phe-stimulated neutrophil (Figures 9-11). By inhibiting activation of protein kinase C, AUR indirectly alters the activity of 5-lipoxygenase while having no apparent effect on cyclooxygenase activity.

C. EFFECTS ON CALCIUM HOMEOSTASIS

The phospholipase C-mediated breakdown of membrane polyphosphoinositides generates a number of metabolites, many of which possess second messenger potential. Of these products, inositol 1,4,5-trisphosphate and its cyclic derivative, inositol 1,2-cyclic-4,5-trisphosphate, have been shown to mobilize calcium in a variety of cellular systems (Berridge and Irvine, 1984). Recently, a soluble 40 kD phosphatase has been isolated from human platelets (Connolly et al., 1985; Connolly et al., 1986a), possessing the ability to hydrolyze the 5-phosphate from inositol 1,4,5-trisphosphate and inositol 1,2-cyclic-4,5-trisphosphate forming inositol 1,4-bisphosphate and inositol 1,2-cyclic-4-bisphosphate, respectively. The newly characterized phosphatase was named inositol 1,4,5-trisphosphate 5'-phosphomonoesterase. The inositol bisphosphates were found to lack calcium mobilizing abilities and were inactive in physiological assays (Streb et al., 1983; Wilson et al., 1985). It was then proposed that the 5'-phosphomonoesterase could function as a signal-terminating step by inactivating the second messenger roles of the inositol phosphates.

When platelets are stimulated by collagen or thrombin, an increased incorporation of $^{32}PO_4$ into proteins of 40 kD and 20 kD is observed (Lyons et al.,

1975; Halsam and Lynham, 1977). The 20 kD protein has been identified as the light chain of platelet myosin (Daniel et al., 1977), however, the identity of the 40 kD protein remained unknown for some time. Various groups have purified the 40 kD protein from platelets (Lyons and Atherton, 1979; Imaoka et al., 1983), and others have demonstrated that it can be phosphorylated *in vitro* in a calcium- and phospholipid-dependent manner, that is, by protein kinase C (Kawahara et al., 1980; Sano et al., 1983). Connolly and coworkers (1986b) were the first to provide direct evidence that protein kinase C phosphorylates the 40 kD protein concomitant with inositol 1,4,5-trisphosphate 5'-phosphomonoesterase activation. This phenomenon has recently been investigated in neutrophils (Della Bianca et al., 1986). The results demonstrate that by priming human neutrophils with non-stimulatory doses of TPA, the increase in inositol 1,4,5-trisphosphate formation and subsequent mobilization of intracellular calcium stores induced by fMet-Leu-Phe, are depressed. Presumably, the 5'-phosphomonoesterase is activated in TPA-primed cells. Interestingly, an enhanced production of superoxide anions is observed suggesting that the stimulation of NADPH oxidase may be linked to protein kinase C activation as opposed to increased levels of calcium in the cytoplasm.

Protein kinase C has also been implicated in another mechanism functioning to maintain calcium homeostasis. When intracellular calcium levels are increased due to cellular activation, both active extrusion and sequestration by calcium-pumping organelles contribute to the recovery of resting calcium levels. TPA has been reported to inhibit the elevation of calcium in many different cells including platelets and neutrophils (Lagaste et al., 1984; MacIntyre et al., 1985; Drummond, 1985). This is thought to occur via the protein kinase C-induced activation of a calcium-transport adenosine triphosphatase in the plasma membrane promoting efflux of calcium from inside the cell.

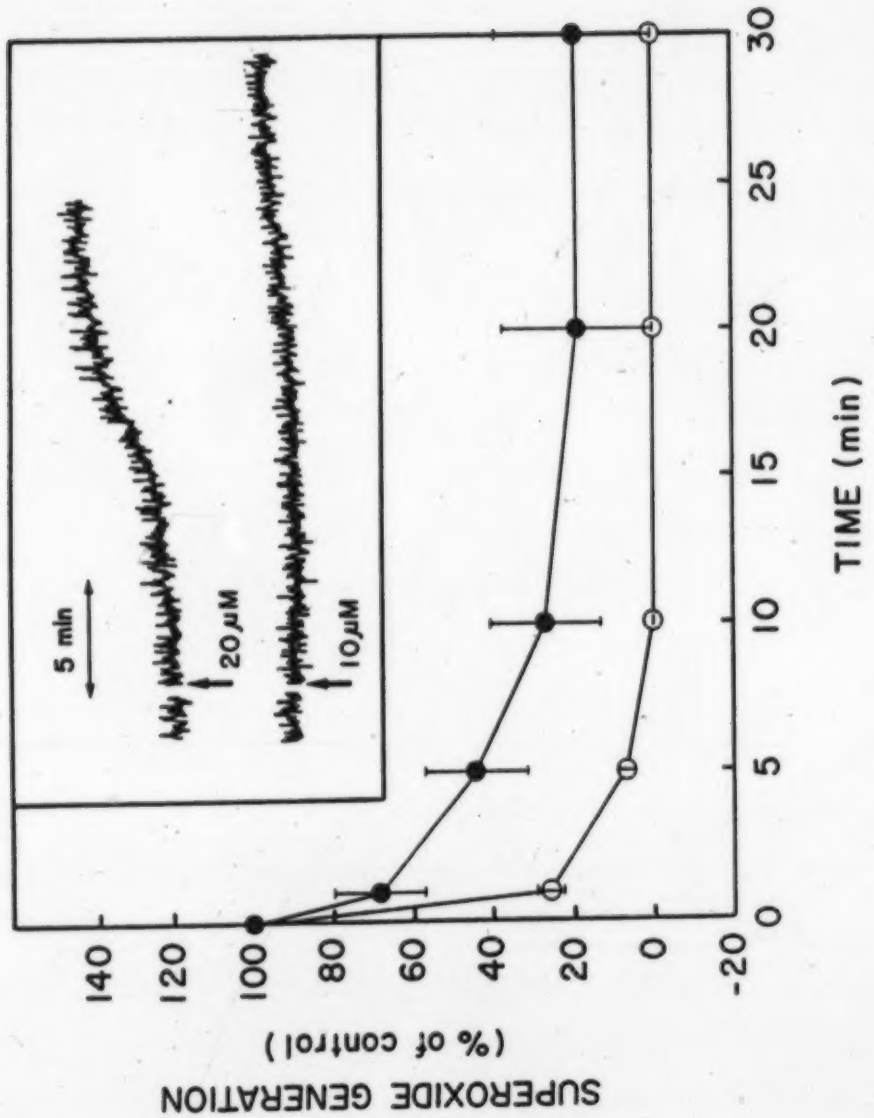
With a possible involvement of protein kinase C in terminating intracellular calcium mobilization and promoting the efflux of calcium under stimulated cell conditions, the effects of AUR on calcium mobilization may be explained. Inhibition of protein kinase C by this gold compound could interrupt activation of inositol 1,4,5-trisphosphate 5'-phosphomonoesterase and plasma membrane calcium-transport adenosine triphosphatase in the stimulated cell, leading to increased intracellular calcium levels. This is supported by the results of Figure 18 where the increase in intracellular calcium induced by fMet-Leu-Phe and F^- is enhanced in the presence of AUR. It is uncertain, however, what

role protein kinase C plays in the regulation of these enzymes under resting cell conditions. If protein kinase C actively maintains low intracellular calcium levels in the absence of a cellular stimulus, then its inhibition would result in increased intracellular calcium levels. This would explain the slow rise in intracellular calcium mediated by the addition of AUR to resting cells (Figure 19). However, since a major component of the AUR-induced rise in intracellular calcium is dependent on the presence of extracellular calcium, ionophore-like effects may also contribute to this drug's action. Further work is indeed necessary to elucidate the mechanism by which AUR increases intracellular calcium concentrations, especially in view of the fact that this drug does not induce overt responses in the neutrophil.

A result of particular importance is the inability of AUR to inhibit the fMet-Leu-Phe- or F^--mediated mobilization of calcium (Figure 18). Figure 36 demonstrates that the increases in intracellular calcium levels induced by 10 and 20 μM AUR were detected after approximately 7 and 12 min, respectively, of cellular exposure to the drug. However, suppression of the fMet-Leu-Phe-mediated respiratory burst was evident after a 1 min exposure to AUR (10 and 20 μM), and was maximal at 15 min. It appears that rises in

Figure 36: The effects of auranofin on neutrophil superoxide anion generation and mobilization of intracellular calcium.

Neutrophils were preincubated in the presence of AUR (● , 10 μM; ○ , 20 μM), for the indicated time intervals. FMet-Leu-Phe (0.1 μM) was added and the rate of superoxide anion generation was calculated and compared to control rates (0.1% ethanol). The results are the mean ± standard error of 5 determinations. Inset: Quin 2 fluorescence measurements in AUR-treated cells performed according to MATERIALS AND METHODS.


SUPEROXIDE GENERATION
(% of control)
140
120
100
80
60
40
20
0
-20
0
5
10
15
20
25
30
TIME (min)
5 min
20μM
10μM

intracellular calcium levels are not necessary for inhibition of the respiratory burst by AUR, but may either be a consequence of this response or an independent event. These data are consistent with the postulate that activation of protein kinase C is sufficient for the stimulation of NADPH oxidase, and the role of calcium in this process remains questionable.

D. INHIBITION OF PHOSPHOINOSITIDE HYDROLYSIS THROUGH PHOSPHORYLATION OF THE GUANINE NUCLEOTIDE-BINDING REGULATORY PROTEIN

Guanine nucleotide-binding regulatory proteins (G-proteins) are a family of transducing units which reside in the plasma membrane of the cell. They function to transmit signals, which arise from agonist binding at the cell surface, to key proteins in the cell involved in signal amplification or transduction. Many reports have implicated a G-protein in the coupling of the fMet-Leu-Phe receptor to phospholipase C activation (Volpi et al., 1983; Yano et al., 1983; Dougherty et al., 1984; Bradford and Rubin, 1985). Recently, various groups have provided evidence for protein kinase C-mediated G-protein phosphorylation and subsequent inhibition of phosphoinositide hydrolysis providing a negative feedback loop in the signal transduction cascade (Labarca et al., 1984; Watanabe et al., 1985;

Katada et al., 1985; Orellana et al., 1985; Lynch et al., 1985; Blackmore and Exton, 1986; Orellana et al., 1987; Yoshimasa et al., 1987). This mechanism appears to only be present during cellular activation and the role of protein kinase C in the regulation of G-protein activity in the resting cell remains to be elucidated.

Recently, Snyder and coworkers (1987 a,b) have demonstrated that AUR, and other gold coordination complexes, stimulate the activity of phospholipase C. Activation was detected in RAW 264.7 macrophages and in situ using purified bacterial phospholipase C. In intact cells, AUR-induced activation of phospholipase C could be explained by an inhibition of protein kinase C, that is, by suppressing G-protein phosphorylation and, therefore, removing inhibition of phospholipase C. This model assumes that basal protein kinase C activity in resting cells maintains a fraction of the G-proteins in the phosphorylated state. In other words, AUR may be removing the protein kinase C-mediated inhibition of phospholipase C subsequent to G-protein phosphorylation. This mechanism may also provide an explanation for the increases in intracellular calcium affected by AUR since phospholipase C activity on inositol phospholipids results in inositol 1,4,5-trisphosphate production. However, this mechanism does not account for the activation of purified phospholipase C induced

by gold compounds. An interesting point to be made is that, in intact cells, maximal stimulation was attained at 0.5 μM AUR, whereas, stimulation of the purified enzyme by AUR was not detected at concentrations lower than 20 μM. One would assume that lower concentrations of the drug would be necessary for activation of the purified bacterial enzyme if the drug was acting at this site directly. However, it appears that this is not the case and what these authors may have observed was non-specific activation of the enzyme at the concentrations of AUR employed in their studies. Since these authors chose to study a mammalian and a bacterial form of phospholipase C, such comparisons between them may not be valid.

IV COMPARISON BETWEEN GOLD COMPOUNDS AND OTHER INHIBITORS OF PROTEIN KINASE C

In many cell types, the existence of endogenous inhibitors of the cyclic adenosine monophosphate-dependent kinase and a variety of phosphatases has been established (McPherson et al., 1979; Ingebritsen and Cohen, 1983). Protein kinase C, in both homogenized and non-disrupted neutrophils, displays very low activity in vitro. Particulate and cytosolic fractions prepared by high speed centrifugation of cell homogenates, exhibit

relatively high protein kinase C activity in comparison (McPhail et al., 1984). Interestingly, recombination of these fractions drastically reduces the enzymatic activity. Chromatographic purification of neutrophil homogenates increases protein kinase C activity (Kraft and Anderson, 1983; Sahyoun et al., 1983). Schwantke and Peuch (1984) reported the existence of a protein inhibitor of protein kinase C in the cytosolic fraction of rat brain homogenates which may be related to the heat-stable, 17 kD calcium-binding protein shown to inhibit the partially purified bovine brain enzyme (McDonald and Walsh 1985 a,b). In the last year, two groups reported the existence of protein kinase C inhibitory activity in the neutrophil cytosol (Balazovich et al., 1986; Huang and Oshana, 1986). This inhibitory activity was found to be absent in homogenates of cytoplasts and specific granule-deficient neutrophils indicating that inhibitory activity may reside in the specific granules of normal neutrophils. Although much more work is necessary to characterize this endogenous inhibitor(s), its presence in the cell may serve to regulate cellular activation by modulating protein kinase C activity.

Apart from the endogenous inhibitors of protein kinase C, many other compounds have been postulated to possess antagonistic activities toward this enzyme. A

variety of agents have the potential to inhibit protein kinase C *in situ*, although many also alter calmodulin activities [trifluoperazine, N-(6-amino-hexyl)-5-chloro-1-napthalene sulfonamide (W-7)]. Mention will be made here of those compounds which have been shown to alter neutrophil responsiveness and a comparison will be made between the activities of these agents and gold compounds.

Hidaka and coworkers developed a selective inhibitor of protein kinase C, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, H-7 (Hidaka et al., 1984; Kawamoto and Hidaka, 1984), which has been shown to alter numerous functions in the neutrophil. None of the studies to date have demonstrated the ability of H-7 to inhibit neutrophil degranulation regardless of the stimulus employed (Berkow et al., 1987). The respiratory burst elicited by TPA was suppressed by H-7, however, the fMet-Leu-Phe-induced response was unaltered (Gerard et al., 1986; Berkow et al., 1987). Interestingly, inhibition was also noted in the A23187-induced respiratory burst (Fujita et al., 1986; Berkow et al., 1987), a process which is thought to be independent of protein kinase C activation. Rises in intracellular calcium levels induced by receptor-mediated stimuli, were attenuated by preincubating the cells with TPA (Sha'afi et al., 1986; Tohmatsu et al., 1986). This was

completely reversed by the addition of H-7 to TPA-pre-treated cells. Endogenous protein phosphorylation mediated by TPA was also suppressed by H-7 (Sha'afi et al., 1986; Fujita et al., 1986). In all of these studies, the H-7 concentrations providing maximal inhibitory activity, were approximately 20 - 50 μM. In another study conducted by Wright and Hoffman (1986), H-7 was inactive as a protein kinase C inhibitor since the neutrophil respiratory burst activated by serum opsonized zymosan, A23187, TPA or fMet-Leu-Phe was insensitive to the presence of H-7 in the reaction mixture. Similar results were obtained by this author (unpublished results). An interesting observation regarding this discrepancy is that Wright and Hoffman, as well as this author, obtained H-7 from commercially-prepared sources. In those studies where H-7 was shown to be an effective inhibitor of protein kinase C, H-7 was either obtained from Dr. Hidaka himself, or was prepared according to his published methods. The reported non-inhibitory effects of H-7 must, therefore, be interpreted with caution.

Another compound displaying antagonistic properties toward protein kinase C, is the antibiotic polymyxin B (Wise et al., 1982; Wrenn and Wooten, 1984; Nel et al., 1985). In the neutrophil, polymyxin B was shown to be a potent inhibitor of degranulation induced by TPA, but

was unable to antagonize the secretory activity of fMet-Leu-Phe. In addition, this compound prevented the inhibition of fMet-Leu-Phe-induced neutrophil degranulation by TPA (Naccache et al., 1985b), a result which provides evidence for the involvement of protein kinase C in regulating G-protein activity.

Tamoxifen is an anticancer agent which has been demonstrated to inhibit protein kinase C in vitro (O'Brien et al., 1985; Su et al., 1985). In the neutrophil, tamoxifen inhibited the TPA- and 1-oleoyl-2-acetylglycerol-induced activation of the respiratory burst with an IC_{50} of approximately 6 μM.

The sphingoid long-chain bases, sphinganine and sphingosine, have recently been shown to inhibit protein kinase C (Hannun et al., 1986). Incubation of neutrophils with sphinganine or sphingosine blocked activation of the respiratory burst triggered by fMet-Leu-Phe, arachidonate, 1-oleoyl-2-acteylglycerol, or TPA. Regardless of the cellular stimulus employed, inhibition was evident at the same concentration of lipid (50 μM) (Wilson et al., 1986). These lipids did not affect the activity of the membrane enzyme complex, NADPH oxidase. The transient rise in intracellular calcium levels elicited by fMet-Leu-Phe was also unaltered. As well, inhibitory concentrations of sphinganine did not significantly affect resting

cytoplasmic calcium levels. Sphinganine did block the TPA-stimulated incorporation of $^{32}PO_4$ into neutrophil proteins in addition to inhibiting the binding of [^{3}H]phorbol 12, 13-dibutyrate to its cytosolic receptor.

In the majority of the neutrophil responses studied, AUR displayed similar inhibitory characteristics as the aforementioned antagonists of protein kinase C. For example, all of the compounds, including AUR, inhibited the activity of protein kinase C in situ as well as the respiratory burst activated by TPA. Inhibition of protein kinase C-mediated endogenous protein phosphorylation was detected with AUR and some antagonists in addition to an alteration in phorbol ester binding to the enzyme present in the cell cytosol. Unlike other protein kinase C inhibitors, AUR increased intracellular calcium levels in intact resting neutrophils, promoted the phosphorylation of a 20-22 kD endogenous protein and inhibited cellular degranulation elicited by receptor-mediated stimuli. In addition, A23187-induced cellular responses were insensitive to AUR. Since the mechanism whereby AUR induces an increase in intracellular calcium levels is unknown, it may be assumed that it, is independent of protein kinase C inhibition due to the fact that the other antagonists of protein kinase C did not possess this property. Therefore, although AUR

appears to share many characteristics with other antagonists of protein kinase C, further studies are necessary to demonstrate that protein kinase C inhibition is the sole mechanism whereby AUR exerts its antiinflammatory effects on the neutrophil.

V GENERAL CONCLUSIONS AND FUTURE RESEARCH

AUR has demonstrated inhibitory activity toward neutrophil degranulation, de novo leukotriene production, the respiratory burst, TPA-mediated protein kinase C redistribution, and TPA-mediated endogenous protein phosphorylation. That these AUR-mediated effects are linked to inhibition of protein kinase C is evidenced by alterations in neutrophil cytosolic and partially-purified platelet protein kinase C activities, and phorbol ester binding, observed in the presence of either AUR or GST. Further evidence of this mode of inhibition by the gold compounds could be obtained by examining the ability of AUR to reverse the TPA-mediated inhibition of inositol 1,4,5-trisphosphate formation and receptor-mediated increases in intracellular calcium levels. GST would be a poor antagonist in these studies since it has difficulty passing through the plasma membrane barrier. However, both gold compounds could be employed in studying their molecular interaction with

purified protein kinase C.

Protein kinase C is a single polypeptide composed of two main regions designated the regulatory and catalytic domains. Protamine sulfate is a protein kinase C substrate which is phosphorylated by the enzyme in a calcium- and phospholipid-independent manner (Takai et al., 1979), unlike histone III-S employed as a substrate in the current research. By implementing this substrate in a protein kinase C assay, and testing the ability of the gold compounds to alter its phosphorylation, information may be obtained on the localization of drug action at the molecular level. For example, the phosphorylation of protamine sulfate occurs solely via the catalytic domain of protein kinase C. If the gold compounds were able to inhibit this reaction, it could be assumed that inhibitory activity is directed at this site in the molecule. However, since the regulatory domain is physically attached to the catalytic domain in these studies, the possibility that the drugs bind to the regulatory domain and exert effects on the catalytic domain allosterically, cannot be ignored. Trypsinization of protein kinase C could overcome these problems. Proteolysis of rat brain protein kinase C <u>in vitro</u> yields two polypeptides, a 50 kD protein kinase active in the absence of calcium and phospholipid, and a 32 kD protein containing binding sites for calcium, phospho-

lipid and diacylglycerol, but lacking in enzymatic activity (Inoue et al., 1977; Takai et al., 1977; Kishimoto et al., 1983b; Tapley and Murray, 1985; Lee and Bell, 1986). By utilizing the methods of trypsinization employed in these studies, a physical separation of the catalytic and regulatory domains of protein kinase C could be accomplished, and a direct action of the gold compounds on either region of the molecule could therefore be elucidated. The results of the current research domonstrate the ability of the gold compounds to alter kinase activity in addition to phorbol ester binding. The observed IC_{50} values for AUR were 0.7 μM and 37 μM for its inhibition of protein kinase C activity and phorbol ester binding, respectively. GST, on the other hand, inhibited both of these activities to the same extent. In view of these results, a clarification of the direct site of inhibition of the gold compounds is necessary.

The differences between the activities of the other inhibitors of protein kinase C and AUR requires further investigation. The results indicate that AUR alters cellular activities secondary to protein kinase C inhibition, with respect to time of onset and concentration required, and that they may be linked to increased intracellular calcium levels.

Human platelet myosin is similar to other myosin

molecules isolated from non-muscle cells. It has a molecular weight of 460,000 and is composed of two heavy chains (200,000) and two different light chains (20,000 and 15,000) (Adelstein et al., 1973). Phosphorylation of the 20,000 molecular weight light chain has been shown to be associated with platelet activation and subsequent release of platelet granular contents (Lyons et al., 1975; Haslam and Lynham, 1977; Lyons and Atherton, 1979; Wallace and Bensusan, 1980). The protein kinase catalyzing this phosphorylation has been identified as the calcium- and calmodulin-dependent enzyme, myosin light chain kinase (Adelstein and Conti, 1975; Nishikawa et al., 1980; Daniel et al., 1981). AUR induced a dose-dependent increase in levels of intracellular calcium, therefore, the possibility exists that this drug could activate myosin light chain kinase since it has been shown to phosphorylate a protein of molecular weight between 20-22,000. Results listed in Table IV, and illustrated in Figures 7 and 8, demonstrate the release of lysozyme in resting cells induced by AUR (1-20 μM) and the enhanced release of both lysozyme and β-glucuronidase by low concentrations of AUR in stimulated cells. Thus, an alternate site of action of AUR may be the indirect activation of myosin light chain kinase via increased intracellular calcium concentrations. [AUR does not alter the activity of

purified platelet myosin light chain kinase (Dr. M. Walsh, personal communication), thereby ruling out a direct action of AUR on the enzyme.] However, before attributing this activity to AUR, many questions must be investigated: (a) Why does GST induce the phosphorylation of the 20-22,000 molecular weight protein and the release of lysozyme in resting cells, but cannot be demonstrated to alter intracellular calcium levels? (b) The presence of TPA appears to block the AUR-induced phosphorylation of the 20-22,000 molecular weight protein, yet the literature is weighted with evidence of TPA-induced phosphorylation of the 20,000 molecular weight protein at an alternate site to myosin light chain kinase (Endo et al., 1982; Naka et al., 1983; Nishikawa et al., 1985; Umekawa et al., 1985; Ikebe et al., 1985). What role does TPA play in modulating AUR-induced phosphorylation of this protein? and (c) Could the increased levels of intracellular calcium induced by AUR explain why A23187-mediated cellular responses are insensitive to the action of this drug? Both agents seem to affect the cell in a similar manner with respect to calcium changes. Investigations of this nature may aid in the understanding of gold drug-induced effects observed in the neutrophil so that further research may be conducted on other cell types involved in the inflammatory process.

VI RELEVANCE OF CURRENT RESEARCH TO RHEUMATOID ARTHRITIS

Effective treatment of rheumatoid arthritis is secondary to an understanding of the sequences of biochemical events which occur within the joints and extra-articular tissues. The etiology of rheumatoid arthritis is unknown and, although many interactive mechanisms are proposed in the disease process (Figure 37), there is no evidence pointing to a salient one. The pathways of Figure 37 do indicate that rheumatoid arthritis may be characterized by extreme proliferation and localized inflammation, involving a variety of cells ranging from peripheral blood platelets to synovial cells of the joint cavity. How it is that gold coordination complexes are able to silence this plethora of activity has been a subject of concern for some time.

Apart from their actions on the neutrophil, gold compounds have been shown to affect other cell types involved in inflammation. Inhibition of monocyte and macrophage effector functions (Persellin and Ziff, 1966; Viken and Lamvik, 1976; Davis et al., 1979; Scheinberg et al., 1982; Harth et al., 1983; Sung et al., 1984), in addition to suppression of myelopoiesis (Hamilton and Williams, 1985), were demonstrated. Evidence supporting an immunosuppressive role for gold drugs came from

Figure 37: The pathophysiology of rheumatoid arthrtis.

IgG, M, A, E - immunoglobulins G, M, A and E

IL1 - interleukin 1

IL2 - interleukin 2

PMN - polymorphonuclear leukocyte

PDGF - platelet-derived growth factor

(Harris, 1985)

Page 220 has been removed because of the inavailability of copyright permission. The material included a comprehensive series of pathways describing the pathophysiology of rheumatoid arthritis taken from Harris, E.D.Jr. in Kelly, W.N., Harris, E.D.Jr., Ruddy, S., Sledge, C.B. (eds.): Textbook of Rheumatology, 2nd Ed. W.B. Saunders and Co., Toronto, 1985, p. 867.

studies which involved cellular interactions of both lymphocytes and monocytes and antibody-mediated immune reactions (Lipsky and Ziff, 1977; Harth et al., 1977; Salmeron and Lipsky, 1982; Petersen, 1984; Griswold et al., 1985). Platelet activities have also been compromised by gold compounds (Kean et al., 1984). A wide range of effects of the gold compounds, as demonstrated by the number of cell types and functions affected, is suggestive of gold drug action at a common regulatory component in these cells. Protein kinase C is a ubiquitous enzyme involved in the transuduction pathway of cellular signals leading to various responses ranging from enzyme secretion to cellular proliferation. Whether drug action is directed at this vital enzyme *in vivo*, remains to be established.

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